

Diploma thesis

**Evaluation of a new multiplex qPCR-based assay
for detection of clinically relevant Aspergillus
species**

submitted by

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for receiving an academic Medical Degree

Doctor of medicine

(Dr. med. univ.)

at the

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Graz, 1.10.2017

I declare that I have written this diploma thesis independently, that I have not used other than the sources/ resources cited, and that I have explicitly marked all material, which has been quoted either literally or by content from the sources used.

Graz, am 1.10.2017

Stefanie Zinke e.h.

Acknowledgements

First of all, I want to thank Univ. Prof. Dr. Harald H. Kessler for the great support, for his time, patience and understanding. He was always available for questions and supported me whenever I needed his advice and supplied ideas and suggestions during the writing process.

I also want to thank Mag.^a Dr.ⁱⁿ Evelyn Stelzl who performed the practical work of this study and Dr. Jürgen Prattes who supported me during the poster presentation.

Finally, I want to thank my family for their great support and for never stop believing in me.

Abstract

Background: Recently, the AspID[®] (OLM Diagnostics, Newcastle, UK) has been introduced. This assay is based on multiplex real-time PCR (qPCR) for detection of clinically relevant *Aspergillus spp.*

Objectives: The analytical performance of the AspID[®] was evaluated by using reference material, the diagnostic performance by testing bronchoalveolar lavage fluid (BALF) specimens from patients with invasive pulmonary aspergillosis (IPA) and those with no evidence for IPA.

Materials and Methods: The analytical performance of the AspID[®] was evaluated utilizing the Quality Control for Molecular Diagnostics (QCMD) 2016 *Aspergillus spp.* DNA External Quality Assurance Program which consisted of 9 sample members. The clinical performance of the AspID[®] was evaluated with 36 BALF specimens obtained from 18 patients with IPA and 18 with no evidence for IPA. Patients were classified as having IPA if the BALF galactomannan (GM) concentration yielded an optical density index (ODI) >3.0 and patients had clinical and radiological findings compatible with IPA. Those without IPA had a BALF GM ODI <0.5 and no clinical and radiological findings compatible with IPA. Patients with and without IPA were matched 1:1 regarding underlying diseases and ICU admission. For detection of *Aspergillus* DNA, samples were extracted using the specific B protocol of the NucliSens[®] easyMAG[™] instrument (bioMérieux, Marcy-l'Etoile, France). The input volume was 400 µl and the elution volume 40 µl. After the lysis step, 4 µl of internal extraction control included in the AspID[®] assay was added. Amplification and detection were performed on the LC 480 II instrument (Roche Diagnostics, Rotkreuz, Switzerland).

Results: When the analytical performance was evaluated, 5 out of 6 *Aspergillus fumigatus* positive samples were identified as positive; however, the assay was not able to detect one panel member correctly that contained *Aspergillus fumigatus* DNA. All *Aspergillus* negative samples were correctly identified as negative. When the clinical performance was evaluated, 20 BALF samples were found to be positive and 14 negative. Two samples (one from a patient with IPA and one from a patient without IPA) showed inhibition and were excluded from analysis. When AspID[®] results were compared to those obtained from GM determination, 29 were found to be concordant and 5 discordant (4 AspID[®]-

positives in patients without IPA and one AspID[®]-negative in a patient with IPA). Sensitivity and specificity for AspID[®] including 95% confidence intervals (CI) were 94.1% (95%CI 73.3 – 99.9) and 76.5% (95% CI 50.1 – 93.2), respectively.

Conclusions: Detection of clinically relevant *Aspergillus spp.* in BALF specimens with the AspID[®] seems to be a promising diagnostic approach in patients at risk for IPA. It may allow early diagnosis and rapid initiation of anti-mold therapy.

Kurzfassung

Hintergrund: Um die klinisch relevante *Aspergillus*-Infektion nachweisen zu können wurde der AspID[®], ein neuer qualitativer Test entwickelt, der auf real-time PCR basiert.

Ziele: In der vorliegenden Studie wurde die analytische Leistung des AspID[®] im Rahmen eines Ringversuches getestet. Für die diagnostische Leistung des Tests wurden Proben aus Bronchoalveolären Lavagen (BAL) von Patientinnen und Patienten untersucht, die an einer invasiven pulmonalen Aspergillose (IPA) erkrankt waren und welche ohne einen Hinweis auf IPA.

Materialien und Methoden: Es wurden Proben von 36 Patientinnen und Patienten untersucht, 18 Patientinnen und Patienten mit und 18 Patientinnen und Patienten ohne einen Nachweis einer pulmonalen Aspergillose. Die Patientinnen und Patienten wurden mit invasiver pulmonaler Aspergillose klassifiziert, wenn der Galactomannan-Test ein Ergebnis von $>3,0$ ergab und wenn klinische und radiologische Ergebnisse gefunden wurden, die mit einer IPA einhergehen. Bei den Patientinnen und Patienten ohne IPA ergab die BAL Probe ein Ergebnis $<0,5$ und es wurden keine klinischen oder radiologischen Hinweise für eine IPA gefunden. Die Patientinnen und Patienten wurden auch hinsichtlich ihrer zugrundeliegenden Erkrankungen miteinander verglichen. Um die *Aspergillus* DNA nachzuweisen, wurde zur Extraktion das specific B protocol des NucliSens[®] easyMAG[™] (bioMérieux, Marcy-l'Étoile, France) verwendet, mit einem Ausgangsvolumen von 400 μ l und einem Eluations-Volumen von 40 μ l. Nach dem Lyse Schritt wurden 4 μ l der internen Kontrolle in der Extraktion hinzugefügt. Die Vervielfältigung und der Nachweis der DNA wurden mit dem LC 480 II Instrument (Roche Diagnostics, Rotkreuz, Switzerland) durchgeführt.

Ergebnisse: Mit dem AspID[®] wurden 20 Proben als positiv und 14 Proben als negativ ausgewertet. Zwei Proben (eine mit und eine ohne IPA) zeigten eine Hemmung und wurden von der Analyse ausgeschlossen. Beim Vergleich der AspID[®]-Ergebnisse mit denen des Galactomannan-Test, konnte bei 29 Proben eine Übereinstimmung gefunden werden und 5 Proben zeigten diskrepante Ergebnisse (vier AspID[®] positive bei Patientinnen und Patienten ohne IPA und eine AspID[®] negative bei einem Patienten/einer Patientin mit IPA). Die Sensitivität, die Spezifität, und die positiv- und negative Wahrscheinlichkeitsrate für den AspID[®], inklusive 95% Konfidenzintervall betrug 94,1% (95%CI 73.3 – 99.9),

76.5% (95% CI 50.1 – 93.2), 4 (95% CI 1.7 – 9.5) und 0.1 (95% CI 0.01 – 0.5).

Zusammenfassung: Der Nachweis von klinisch relevantem *Aspergillus* aus BAL Proben mit dem AspID[®] scheint eine vielversprechende diagnostische Methode zu sein, vor allem bei Patientinnen und Patienten die ein Risiko haben eine invasive pulmonale Aspergillose zu entwickeln. Er könnte die frühe Diagnose und die damit verbundene rasche Einleitung einer geeigneten Therapie ermöglichen.

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1. Introduction

1.1 Epidemiology

Aspergillus is a genus consisting of a few hundred mold species found worldwide. It was first described in 1729 by Pier Antonio Micheli, an Italian priest and biologist who was one of the first people to study the kingdom of fungi. Micheli refused the idea of "spontaneous generation" by showing that fungal spores grown on a medium will generate the same kind of fungus. The shape of *Aspergillus* reminded him of an aspergillum (from the Latin *aspergere* = to scatter), a device used for sprinkling holy water during a liturgical service (1). The shape of a typical *Aspergillus* spp. is shown in Fig. 1.

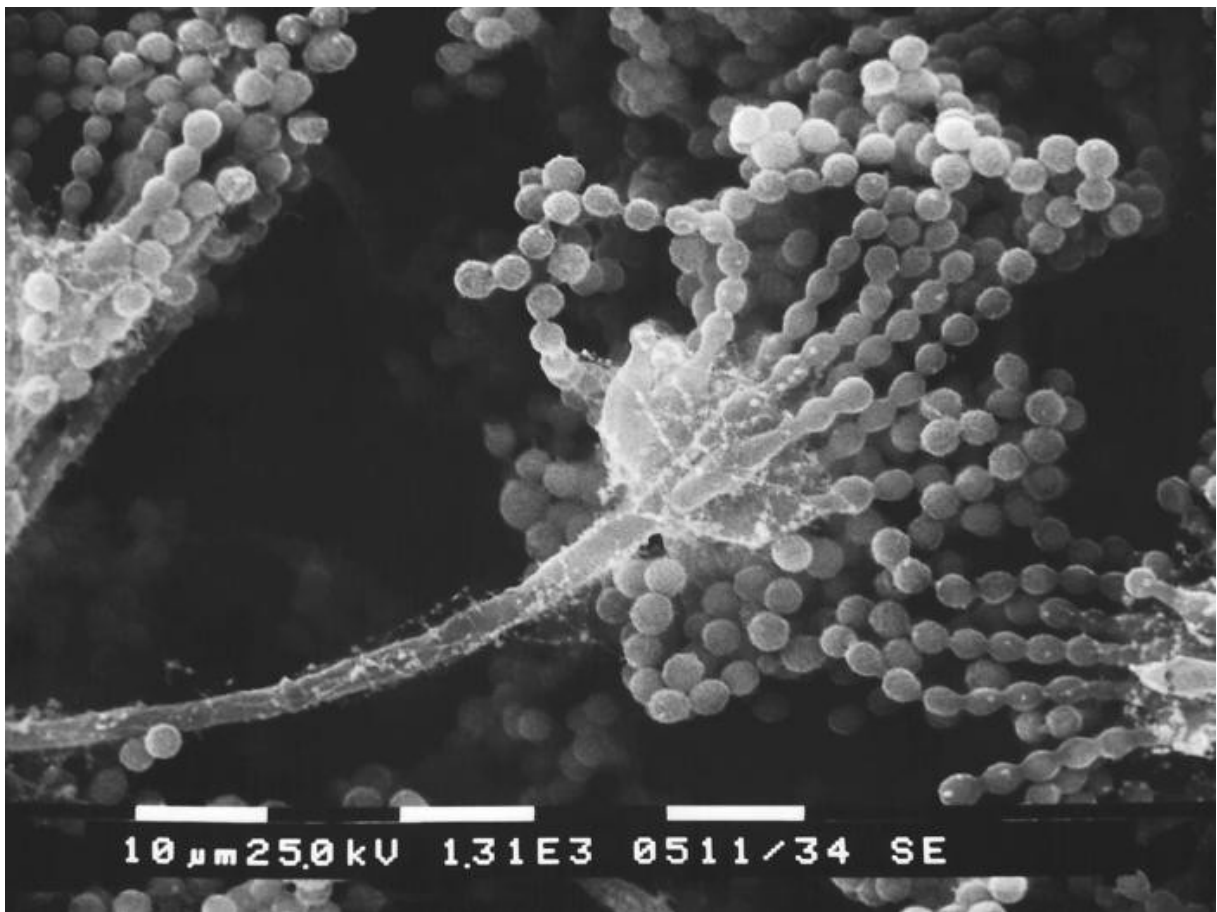


FIG.1. Conidiophore of *Aspergillus fumigates* (taken from <https://phil.cdc.gov/phil/home.asp>).

Aspergillus spores are present in the air we breathe; therefore, the human airways are exposed constantly to the inhalation of several hundred spores. Usually, these spores are harmless for immunocompetent patients having several mechanisms and pattern recognition systems such as alveolar macrophages, neutrophils, and antimicrobial peptides against *Aspergillus* (2). The innate immune system has also mechanisms to remove *Aspergillus* from the airway including the physical and mechanical barriers of the respiratory tract, phagocytic cells, antimicrobial peptides, and receptors that are able to recognizing *Aspergillus* (4). Only in people with a weakened immune system, damaged lungs or with allergies, *Aspergillus* can cause disease. *Aspergillus* infections include invasive aspergillosis, allergic bronchopulmonary aspergillosis, chronic pulmonary aspergillosis, and aspergilloma. High numbers of *Aspergillus* spores are found with air conditioning units, composting facilities, damp ground, flood damaged houses, or even hospital buildings (3).

The spectrum of aspergillosis includes also extrapulmonary infections, such as cutaneous manifestations, otomycosis, and endocarditis. Patients after hematopoietic stem cell transplantation and patients with leukemia are high risk patients to acquire invasive pulmonary aspergillosis. *Aspergillus fumigatus* seems to be the predominant agent of invasive pulmonary aspergillosis (IPA), followed by *Aspergillus terreus* or *Aspergillus flavus*, depending on different studies (4).

1.2 *Aspergillus spp.*

More than 200 different species of *Aspergillus* have been described worldwide; only 10% are clinically relevant as infectious agents. The wide spectrum of disease has been explained by different reaction of the individual human immune system (2).

Many outstanding morphological characteristics of *Aspergillus* species have been described; the combination of those characteristics is responsible for an successful pathogenic potential (5).

1.2.1 *Aspergillus fumigatus*

The human pathogen *Aspergillus fumigatus* is the most causative agent of human aspergillus infections. The interaction between host and fungus contributes to the pathogenesis of *A. fumigatus*. The progression of disease is a result of the fungal growth and the immune response of the host (6).

The pathogenic potential of *Aspergillus fumigatus* depends on the stage of the infectious life cycle (**Fig. 2**). It starts with the production of asexual spores, called conidia. They are distributed in the air and with the inhalation of these airborne conidia the primary way of infection has started and taken place in the bronchioles or alveolar spaces (6).

The biology of *Aspergillus fumigatus* shows a unique combination of features that lead to pathogenicity. The average size of *Aspergillus fumigatus* conidia is 2 to 3 μm , ideal for infiltrating deep into the alveoli, in contrast to other human pathogens including *Aspergillus flavus* and *Aspergillus niger* with larger conidia, which can be removed more easily by mucociliary clearance in the upper respiratory tract (6). *Aspergillus fumigatus* is more thermotolerant than other *Aspergillus spp.*, it grows well at 37° C and can stand temperatures above 50° C (6).

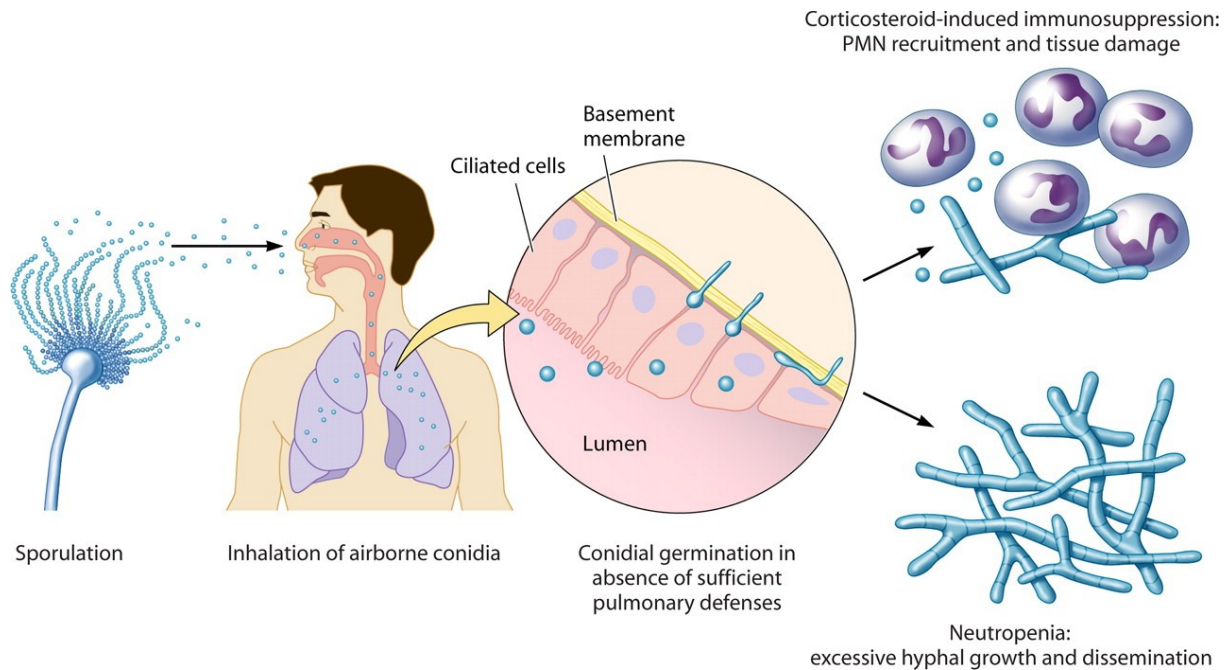


FIG. 2. Infectious life cycle of *Aspergillus fumigatus*(taken from Dagenais TRT, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. Clin Microbiol Rev. 2009 Jul;22(3):447–65).

1.2.2 *Aspergillus terreus*

Nosocomial infections with *Aspergillus terreus* seem to increase worldwide (**Fig. 3**). Treatment of *Aspergillus terreus* is more difficult in comparison to other *Aspergillus spp.*; *Aspergillus terreus* is resistant against the antifungal drug amphotericin B. Furthermore, *Aspergillus terreus* infections are disseminated easier in humans and have a greater lethality (7). Because *Aspergillus terreus* produces a secondary metabolite called lovastatin, the potent drug for lowering blood cholesterol levels, this organism is also used for production of lovastatin(8).



FIG. 3. *Aspergillus terreus*. J Scott, *Aspergillus terreus* colony on Rose Bengal Agar, photographed in Nomarski Differential Interference Contrast Microscopy (taken from https://en.wikipedia.org/w/index.php?title=Aspergillus_terreus&oldid=764705871).

1.3 Aspergillosis

The disease caused by *Aspergillus spp.* is called aspergillosis. The spores of this common mold are found everywhere in the environment. They are inhaled in large numbers every day. In healthy people, the immune system can easily manage with *Aspergillus* spores; however, people with immunosuppression and those with underlying chronic pulmonary disease have an increased risk to become seriously sick.

There are different types of aspergillosis with some types proceeding milder; for instance, an excessive immune response which produces allergic bronchopulmonary aspergillosis leading to the formation of an aspergilloma in case of a competent immune system. However, infections may also lead to invasive pulmonary aspergillosis with high mortality, especially in immunocompromised patients (2).

1.3.1 Invasive pulmonary aspergillosis (IPA)

IPA is the most common fungal pulmonary infection in immunocompromised patients (**Fig. 4**). Patients mostly affected include hematopoietic stem cell transplant recipients and patients with hematological malignancies during chemotherapy. The survival of patients with IPA is very poor because of difficulties with early diagnosis and the lack of effective treatment options. Diagnostic techniques must be improved regarding earlier detection. Therefore, high resolution computer tomography of the chest and sequential monitoring of *Aspergillus* antigen and/or *Aspergillus* DNA are helpful tools(9). The mortality rate is particularly high in transplant patients with 70%. It is significantly lower in patients undergoing allogeneic stem cell or solid organ transplantation with up to 15% (2).

IPA is usually induced by *Aspergillus fumigatus* with clinical consequences including pneumonia, tracheobronchitis, and pleural effusion(2). The causative pathogen may invade the pulmonary arteries, causes hemorrhagic infarctions, and spreads hematogenously predominantly to the brain, gastrointestinal tract, and other organs. Vessels affected may be closed in a thrombotic or a thromboembolic way and hemorrhagic necrosis also possible (10).

The most common symptoms of IPA are nonspecific such as fever, chest pain, dyspnea, cough, and hemoptysis, which make the clinical diagnosis even more difficult. Serological studies and radiology testing are nonspecific but of increasing importance for a safe diagnosis.



FIG 4. Bronchopulmonary angioinvasive aspergillosis (taken from <http://pictures.doccheck.com/de/photo/17804-bronchopulmonale-angioinvasive-aspergillose>).

1.3.2 Aspergilloma

The classical aspergilloma is a conglomeration of condensed hyphae in preexisting cavities, for example, in the maxillary sinus, tuberculous cavern, or bronchiectasis (**Fig. 5**). It develops over different stages and in the late stage, it may be a source for an allergic bronchopulmonary aspergillosis(10). Most patients lack symptoms but there is another life-threatening form with episodes of hemoptysis explained by hyphal invasion of the bronchial arteries(2).

The association between aspergilloma and allergic bronchopulmonary aspergillosis is still unclear. The life-threatening hemoptysis can be prevented by surgery and is still the first choice, while the bronchial artery embolization is state-of-the-art treatment for patients with hemoptysis who are unfit for surgical resection. Another new therapy approach is the instillation of antifungals such as amphotericin B, which may resolve the aspergilloma in some cases completely(2).

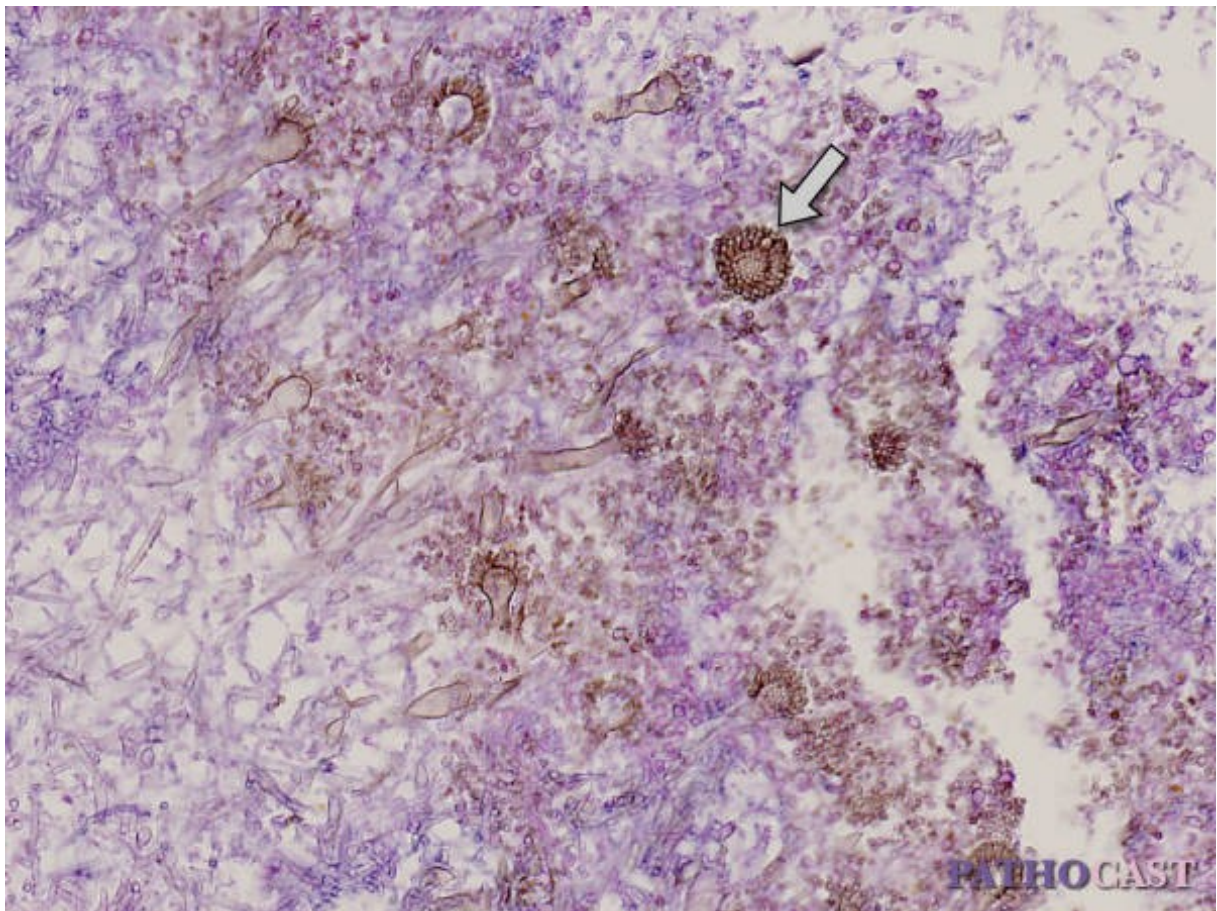


FIG 5. Histology of aspergilloma in the lung with conidiophores (taken from <https://pictures.doccheck.com/de/photo/18507-aspergillus-fumigatus>).

1.3.3 Allergic Bronchopulmonary Aspergillosis (ABPA)

ABPA is an allergic form of aspergillosis with a complex reaction of the immune system and may include Type I, Type III, and Type IV reactions (**Fig. 6**). ABPA may lead to bronchiectasis and fibrosis. Patients with cystic fibrosis have a greater risk to develop ABPA(10).

Consequences of pulmonary aspergillosis include T-lymphocyte activation, cytokine and immunoglobulin release, and inflammatory cell recruitment. Local inflammation may lead to mucus production, airway hyper-reactivity, and bronchiectasis ultimately(11).

Pre-existing chronic pulmonary disease including asthma, bronchiectasis, chronic-obstructive pulmonary disease, cystic fibrosis, and immunodeficiency caused by chronic granulomatous disease or hyper-IgE syndrome can accelerate by ABPA. In such cases, clinical symptoms are very similar to those caused by an infective bacterial exacerbation and therefore, diagnostic criteria have been established to differentiate between ABPA and infective exacerbation in patients with cystic fibrosis, nevertheless clinical difficulties are still present (2).

There is not just one single test to diagnose ABPA, nor are there general criteria, but there are helpful and widely accepted diagnostic criteria. A combination of clinical symptoms, radiographic imaging, and serological tests are used to diagnose ABPA (11).



FIG 6. Manifestation of ABPA (taken from <http://pictures.doccheck.com/de/photo/44744-bronchien-aspergillose-praeparat>).

1.4 Diagnostic approaches of IPA

The identification of clinically relevant *Aspergillus spp.* has been important for an appropriate antifungal therapy. Diagnostics of IPA is based on pulmonary computer tomography (CT) scan findings and non-culture based laboratory tests including galactomannan and DNA detection in blood or bronchoalveolar lavage (BAL) samples (12). Furthermore, rapid bedside diagnosis of invasive aspergillosis has been introduced recently (13).

Galactomannan (GM) is a component of the cell wall of *Aspergillus spp.* It is released during the fungal growth and can be detected in patients with invasive aspergillosis. The assay is performed with monoclonal antibodies in an enzyme-linked immunosorbent assay (ELISA) (13).

Polymerase chain reaction (PCR) is the method of choice for early and rapid diagnosis of invasive aspergillosis. It also has the ability to identify opportunistic molds. Fungal DNA can be detected at a very early stage of disease. The sensitivity of PCR is excellent, but there may be specificity problems due to contaminations (12). Today, in the routine diagnostic laboratory, molecular assays based on real-time polymerase chain reaction (qPCR) have largely replaced those based on conventional PCR (14).

A newly introduced rapid bedside assay uses the secondary metabolites produced from *Aspergillus* in the patients' breath. This assay may be especially important for diagnosis of invasive aspergillosis in high risk patients with a sensitivity and specificity over 90% (13).

1.5 Treatment

Morbidity and mortality caused by invasive aspergillosis are increasing. The number of patients with malignancies and those undergoing allogeneic hematopoietic stem cell or organ transplantation treated with massive immunosuppressive therapy is increasing as well as their improved survival from formerly fatal bacterial infections. Early initiation of effective systemic antifungal treatment is essential for a successful clinical outcome in these patients(10).

The gold standard of systemic treatment of invasive aspergillosis is voriconazole, with liposomal amphotericin B and isavuconazole as alternatives. Other triazole agents (e.g., posaconazole and itraconazole), amphotericin B lipid complex, and echinocandins are alternatives when primary therapy fails. Combination of antifungal drugs is not investigated so far (13). Treatment must be initiated immediately when clinical signs of invasive aspergillosis are suggested. In case of treatment failure, antifungal therapy may be changed (13).

Prophylaxis with voriconazole or posaconazole against *Aspergillus spp.* may be worth considering to prevent invasive aspergillosis especially in patients with neutropenia (12). After lung transplantation, patients should be treated with a systemic triazole or inhale amphotericin for 3 to 4 months. These recommendations are based on observational studies; treatment in patients with other organ transplantations must be chosen individually (13).

Adverse drug effects of azoles include electrocardiographic changes, especially QT prolongation, and photosensitivity, especially when administering voriconazole, with a significant increase of the risk to develop skin cancer in immunocompromised patients. Furthermore, triazoles are strong inhibitors of cytochrome P450. Interactions with other drugs involved in the cytochrome P450 metabolism must thus be taken into consideration. Finally, because antifungal therapy became more and more common within recent years, the possible development of multi-drug resistance must be observed carefully (13).

2. Objectives

The aim of this study was to evaluate the analytical and clinical performance of a new kit, the AspID[®] assay (OLM Diagnostics), based on multiplex qPCR for the identification of clinically relevant *Aspergillus spp.* with simultaneous detection of *Aspergillus terreus*. The analytical performance of the AspID[®] was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2016 *Aspergillus spp.* DNA External Quality Assurance Program. The clinical performance of the AspID[®] was evaluated with 36 BAL specimens obtained from 18 patients with IPA and 18 with no evidence for IPA.

3. Materials and Methods

3.1 Study design

3.1.1 Analytical performance

The accuracy of the AspID[®] was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2016 *Aspergillus spp.* DNA EQA Programme. The panel consisted of nine members including *Aspergillus conidia* or *Aspergillus fumigatus* and negatives. Characteristics of the panel is shown in Table 1.

Table 1 Characteristics of the panel

Vial no.	Sample content	Matrix
1	Negative	TE buffer
2	<i>Aspergillus fumigatus</i> DNA	TE buffer
3	<i>Aspergillus fumigatus</i> DNA	TE buffer
4	<i>Aspergillus fumigatus</i> DNA	TE buffer
5	<i>Aspergillus fumigatus</i>	Synthetic sputum
6	Negative	Synthetic sputum
7	<i>Aspergillus fumigatus</i>	Synthetic sputum
8	Negative	Plasma
9	<i>Aspergillus fumigatus</i>	Plasma

3.1.2 Clinical performance

Thirty-six BALF specimens obtained from 18 patients with IPA and 18 with no evidence for IPA were studied (Table 2). Patients were classified as having IPA when BALF galactomannan (GM) determination yielded an optical density index (ODI) >3.0 and patients had clinical and radiological findings compatible with IPA. Those without IPA had a BALF GM ODI <0.5 and no clinical/radiological findings compatible with IPA. Patients with and without IPA were matched 1:1 regarding underlying diseases and ICU admission.

Table 2: Characteristics of patients with IPA and without IPA

	Patients with IPA	Patients without IPA
Age, years (age range)	64.5 (48 – 84)	66.5 (25 – 81)
Female	10	9
Risk factors for IPA*		
Hematological malignancy	3	3
ICU admission	12	13
Solid tumor	4	4
Autoimmune disease	2	2
BALF GM levels, ODI (range)	6.9 (3.2 – 25)	0.1 (0.1 – 0.3)
<i>Aspergillus</i> positive BALF culture	7	0

*A patient may have more than one underlying risk factor.

3.2 Methods

3.2.1 Extraction of nucleic acids

Nucleic acid extraction was performed on the NucliSens[®]easyMAG[™] platform (bioMérieux, Marcy-l'Étoile, France; Fig. 7). This instrument uses the magnetic particle technology for capture of nucleic acids. All samples used in this study were extracted with the NucliSens[®]easyMAG[™] accessory products (bioMerieux) using the specific B protocol. The input volume was 400 µl. After the lysis step, 4 µl of internal extraction control (IEC) included in the AsplD[®] (OLM Diagnostics, Newcastle upon Tyne, United Kingdom) assay was added. The IEC is added to distinguish true negative samples from false negative samples, which can result from nucleic acid degradation, failure of nucleic acid extraction step, PCR inhibition or qPCR instrument malfunction. The primers and probe necessary to detect the IEC are included in the multiplex primer and probe mix. The IEC gives a quantification cycle value of 28+/-3, with the variation depending on the efficiency of sample extraction and level of sample dilution.

The extracted DNA was eluted automatically with 40 µl of elution buffer.



FIG.7.The NucliSens®easyMAG™ platform (picture taken by Stefanie Zinke on April 20, 2017).

3.2.2 Amplification and detection

Nucleic acid extracts were amplified and detected with the AspID® on the Light Cycler® 480 II instrument (Roche Diagnostics, Rotkreuz, Switzerland). With the AspID®, individual primer and probe designs for detection of clinically relevant *Aspergillus spp.* in general, as well as *Aspergillus terreus* in particular, have been combined into a single assay and their DNA can be detected through the different fluorescent channels as described in the manufacturer's package insert. AspID® makes use of the qPCR chemistry, which is based on the detection of light emitted by hydrolysis probes (Fig.8).

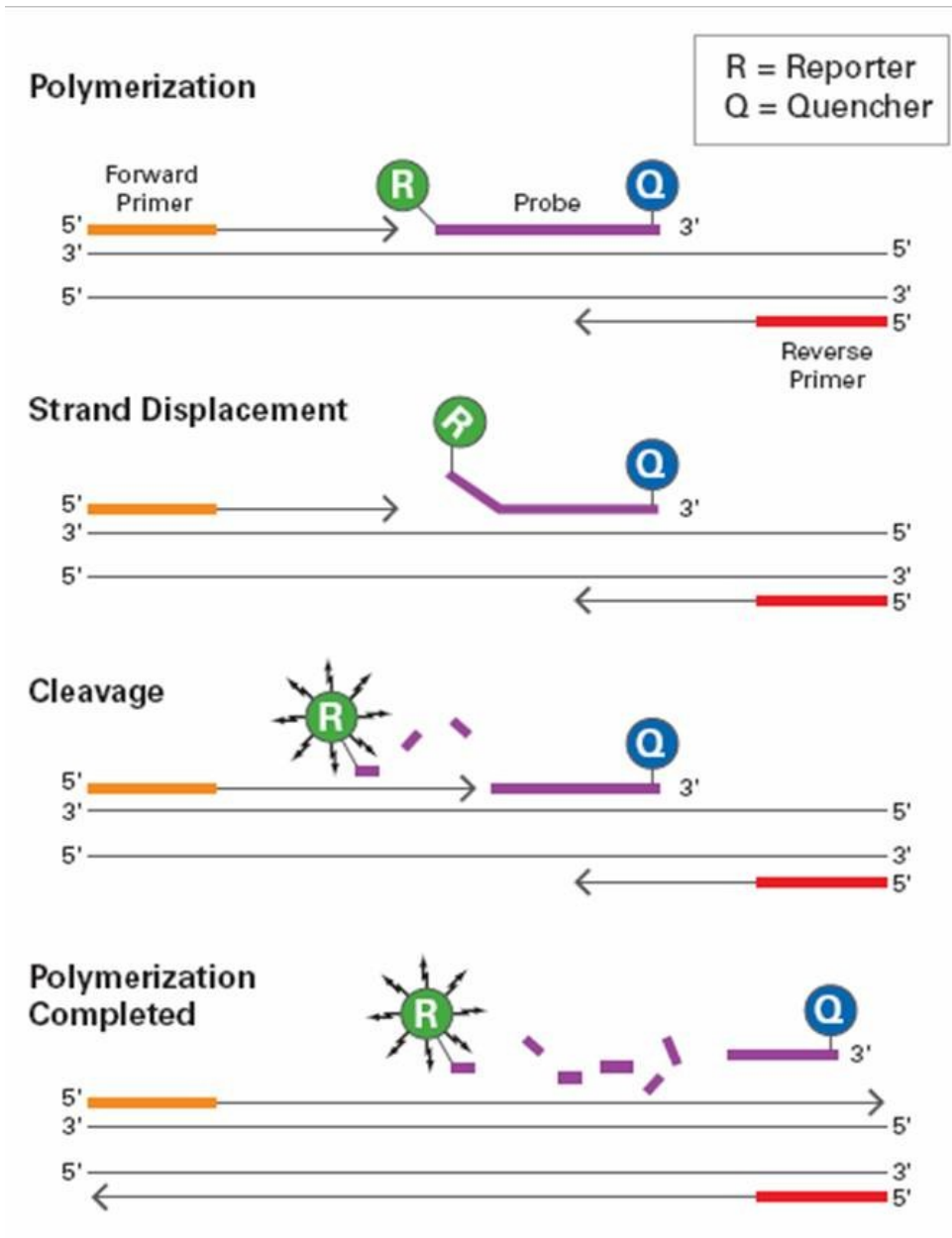


FIG. 8. Principle of the hydrolysis probe-based assay (taken from <https://es.wikipedia.org/w/index.php?title=TaqMan&oldid=97268506>).

The AspID[®] kit also contains a positive control tube with templates for each of the two assay targets (*Pan-Aspergillus* and *A. terreus*). The positive control is handled like a normal nucleic acid extract and indicates that the primers and probes for detecting *Aspergillus* targets are working properly in the run. Its use is recommended in every run as it provides more confidence in results if all samples are negative. The positive control does not need to be subjected to a nucleic acid extraction procedure. Care should be taken during its resuspension from the

lyophilised state to ensure that it does not contaminate any other kit component, which would lead to false-positive results. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. Any risk can be minimized by sealing all other samples and negative controls before pipetting the positive control well.

To confirm the absence of contamination, at least one no template control (NTC) reaction must be included in every PCR run. For this reaction, RNase/DNase free water should be used instead of template.

For qPCR and detection with the AspID[®], 7 µl of the master mixture and 3 µl of the extracted sample were pipetted into a well of a PCR plate. After sealing the plate, amplification and detection were performed on the Light Cycler[®] 480 II CE/IVD (Roche Diagnostics, Penzberg, Germany) instrument (Fig. 9).

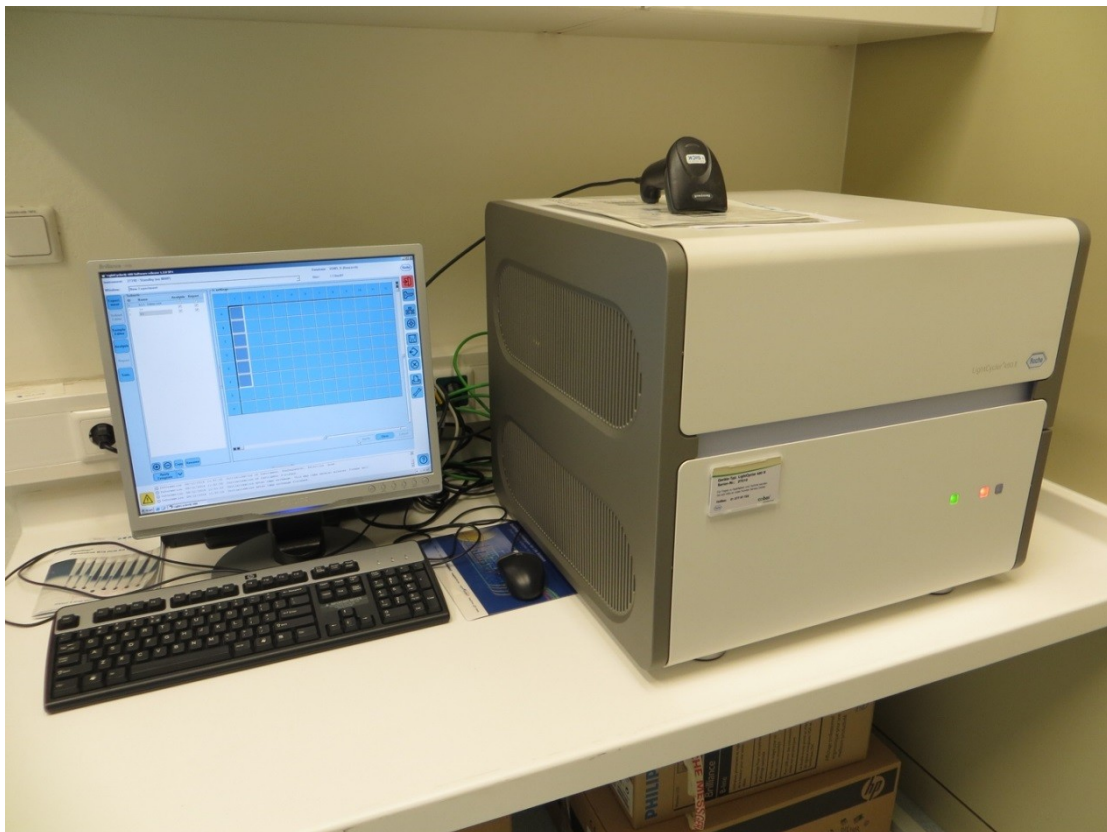


FIG. 9. The Light Cycler[®] 480 II instrument (picture taken by Stefanie Zinke on April 20, 2017).

4. Results

4.1 Analytical performance

When accuracy was determined, five out of six *Aspergillus fumigatus* positive samples were identified as positive; however, the assay was not able to detect one panel member correctly that contained *Aspergillus fumigatus* DNA (Table 3). All *Aspergillus* negative samples were correctly identified as negative.

Table 3: Accuracy testing utilizing the Quality Control for Molecular Diagnostics (QCMD) 2016 *Aspergillus spp.* DNA EQA Programme.

Vial no.	Sample content	Matrix	Result expected	Result obtained
1	Negative	TE buffer	Negative	Negative
2	<i>Aspergillus fumigatus</i> DNA	TE buffer	Positive	Positive
3	<i>Aspergillus fumigatus</i> DNA	TE buffer	Positive	Positive
4	<i>Aspergillus fumigatus</i> DNA	TE buffer	Positive	Negative
5	<i>Aspergillus fumigatus</i>	Synthetic sputum	Positive	Positive
6	Negative	Synthetic sputum	Negative	Negative
7	<i>Aspergillus fumigatus</i>	Synthetic sputum	Positive	Positive
8	Negative	Plasma	Negative	Negative
9	<i>Aspergillus fumigatus</i>	Plasma	Positive	Positive

4.2 Clinical Performance

With the new AspID[®], 20 BALF samples were found to be positive and 14 negative (Fig. 10). Two samples showed inhibition and were excluded from analysis. When AspID[®] results were compared to those obtained from GM determination, 29 were found to be concordant and 5 discordant (four AspID[®]-positives in patients without IPA and one AspID[®]-negative in a patient with IPA). Sensitivity, specificity, positive- and negative likelihood ratio for AspID[®] including 95% confidence interval (CI) were 94.1% (95%CI 73.3 – 99.9), 76.5% (95% CI 50.1 – 93.2), 4.0% (95% CI 1.7 – 9.5), and 0.1 (95% CI 0.01 – 0.5), respectively.

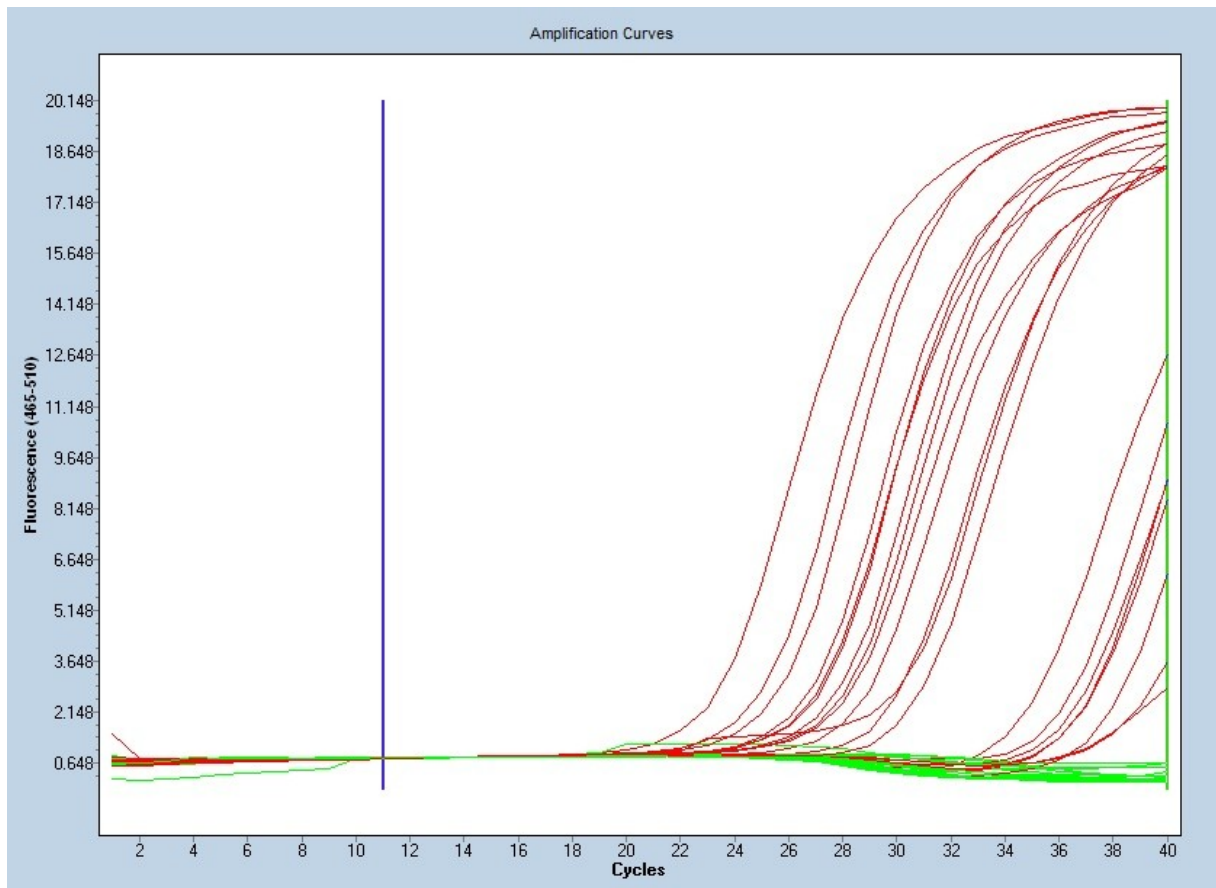


FIG.10. Fluorescence curves obtained from 20 BALF samples that were found to be positive for *Aspergillus spp.* DNA (red) and 14 BALF samples that were found to be negative (green).

5. Discussion

Molecular assays for measurement of *Aspergillus* DNA have become an important tool for the diagnosis of *Aspergillus* associated disease. Rapid and accurate detection of *Aspergillus* DNA has been shown to have potential for improving the diagnosis allowing rapid therapeutical intervention leading to an improved outcome of infections with *Aspergillus* species. Molecular tests for *Aspergillus* have been limited historically by lack of standardization and variable sensitivities and specificities (14).

The AspID[®] is based on real-time PCR and has been designed for detection of clinically relevant *Aspergillus* species with simultaneous identification of *Aspergillus terreus*. In this study, this new qualitative assay was evaluated and results were compared to those obtained with a galactomannan assay. For detection and quantitation of *Aspergillus* DNA, any sample material can be used. Blood, respiratory, and tissue specimens have been used predominantly for direct detection of *Aspergillus*(14). An important limitation of testing respiratory specimens is the inability to differentiate airway colonization from invasive disease on the basis of nucleic acid detection alone (14). Nevertheless, in this study, the analytical and clinical performance of the AspID[®] kit was evaluated by using 36 BALF specimens.

The accuracy of the AspID[®] was tested utilizing the Quality Control for Molecular Diagnostics (QCMD) 2016 *Aspergillus spp.* DNA EQA Programme. The panel consisted of nine members including *Aspergillus conidia*, *Aspergillus fumigatus*, and negatives. Five out of six *Aspergillus fumigatus* positive samples were identified as positive; however, the assay was not able to detect one panel member correctly that contained *Aspergillus fumigatus* DNA. For this panel member, the samples status was stated as “educational” indicating a low pathogen concentration. Unfortunately, QCMD does not indicate the exact pathogen concentrations in single panel members. Of all positive members, this member was reported correctly by the lowest number of laboratories participating in this EQA challenge. All *Aspergillus* negative samples were correctly identified as negative.

In the clinical study, two samples showed inhibition and were excluded from analysis. Molecular assays may be impacted by inhibitors at some time. This problem exists especially with the wide range of non-blood specimens often used for detection of pathogens. Additionally, suboptimal sampling conditions may make PCR based assays for pathogen detection in non-blood specimens especially vulnerable. Inhibitors generally exert their effects through direct interaction with DNA or interference with DNA polymerases. Common specimen types known to contain inhibitors include blood, sputum, urine, feces, and tissues. Additional sources of inhibitors may be materials and reagents that are exposed to samples during processing or DNA purification. The best way to avoid PCR inhibition is to prevent the inhibitor from being processed with the sample; however, dilution of the sample may also help (15). PCR inhibitors may interact with different steps of PCR analysis, especially DNA may adsorb to polymeric surfaces, to the wall of vessels and reaction tubes, during sample processing, extraction or during PCR. There are many PCR inhibitors that target the DNA polymerase directly or indirectly. The exact mechanism of PCR inhibitors is still unknown so far (16). PCR inhibitors are a heterogeneous class of substances that act at different steps of the diagnostic procedure. They are present in a large variety of sample types and may lead to decreased PCR sensitivity or even false-negative PCR results. Several strategies have been developed to remove PCR inhibitors during sample preparation. However, it cannot be guaranteed that the preparations are free of PCR inhibitors, all reactions should be analyzed for the presence of inhibitory effects. The development of standardized controls has therefore been recommended for evaluation of diagnostic PCR results and for comparison of the efficiency of different PCR protocols (16).

In conclusion, detection of clinically relevant *Aspergillus spp.* in BALF specimens with AspID[®] PCR reagents seems to be a promising diagnostic approach in patients at risk for IPA. It may allow early diagnosis and rapid initiation of anti-mold therapy.

6. Annex

Results from this study were presented at the ÖIK – Österreichischer Infektiologie Kongress, Saalfelden, 29.3. – 1.04.2017 (S. E.-M. Zinke, J. Prattes, M. Hoenigl, S. Heldt, S. Eigl, G. L. Johnson, S. Bustin, E. Stelzl, H. H. Kessler: Evaluation of a new multiplex qPCR kit for detection of clinically relevant *Aspergillus spp.* in BAL fluid).

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8. List of figures

FIG. 1. The conidiophore of the fungal organism *Aspergillus fumigatus*. Taken from the CDC [<http://phil.cdc.gov/phil/home.asp> Public Health Image Library]. Image credit: CDC/Dr. Libero Ajello (PHIL #4297), 1963. == Licensing == {{PD-USGov-HHS-CDC}}

FIG. 2. Infectious life cycle of *Aspergillus fumigates* [cited 2017 Jul 5]. Taken from:Dagenais TRT, Keller NP. Pathogenesis of *Aspergillus fumigatus* in Invasive Aspergillosis. *ClinMicrobiol Rev.* 2009 Jul;22(3):447–65.

FIG. 3. *Aspergillus terreus*. In: Wikipedia [Internet]. 2017 [cited 2017 Mar 12]. Taken from:https://en.wikipedia.org/w/index.php?title=Aspergillus_terreus&oldid=764705871

FIG. 4. GmbH DMS. BronchopulmonaleangioinvasiveAspergillose [Internet]. DocCheck Pictures. [cited 2017 Mar 6]. Taken from: <http://pictures.doccheck.com/de/photo/17804-bronchopulmonale-angioinvasive-aspergillose>

FIG 5. Histology of aspergilloma in the lung with conidiophores GmbH DMS. *Aspergillus fumigatus* [Internet]. DocCheck Pictures. [cited 2017 Jul 5]. Taken from: <https://pictures.doccheck.com/de/photo/18507-aspergillus-fumigatus>

FIG. 6. Bronchien Aspergillose (Präparat) - DocCheck Pictures [Internet]. [cited 2017 Jul 5]. Taken from: <http://pictures.doccheck.com/de/photo/44744-bronchien-aspergillose-praeparat>

FIG. 7. The NucliSens®easyMAG™ platform.

FIG. 8. Principle of the hydrolysis probe-based assay. Taken from: TaqMan. In: Wikipedia, la enciclopedia libre [Internet]. 2017 [cited 2017 Jul 7]. Taken from: <https://es.wikipedia.org/w/index.php?title=TaqMan&oldid=97268506>

FIG. 9. The Light Cycler® 480 II instrument.

FIG. 10. Fluorescence curves obtained from 20 BALF samples that were found to be positive for *Aspergillus spp.* DNA (red) and 14 BALF samples that were found to be negative (green).