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Mycology

Evaluation of a PCR-electrospray ionization mass spectrometry platform for detection and identification of fungal pathogens directly from prospectively collected bronchoalveolar lavage specimens

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ABSTRACT

The incidence of invasive fungal infections is on the rise worldwide due to the growth of the immunocompromised population. We report here the use of a diagnostic assay that utilizes a universal extraction method, broad spectrum PCR amplification and analysis via electrospray ionization mass spectrometry (PCR/ESI-MS) to detect and identify more than 200 pathogenic fungi directly from bronchoalveolar lavage (BAL) specimens in less than 8 hours. In this study, we describe both analytical and clinical performance of the assay, when run with prospectively collected clinical BAL specimens. In 146 patients with probable and possible fungal infections defined by EORTC/MSG (European Organization for Research and Treatment of Cancer/Mycoses Study Group) criteria, the PCR/ESI-MS assay demonstrated a sensitivity of 90.9% (95% CI: 76.4–96.9%) and a specificity of 82.3% (95% CI: 74.2–88.2%). This data demonstrates the utility of a non-culture based broad fungal targets molecular diagnostic tool for rapid and accurate diagnosis of invasive fungal infections in patients at risk of developing fungal diseases.

1. Introduction

The diagnosis of fungal infections is clinically challenging and often relies on a combination of clinical, radiologic, and microbiological factors. Traditional method of culture-based fungal identification is a time consuming process with some fungi requiring up to 6 weeks of growth before identification can be made. Such lengthy turn-around-times do not meet the clinical need for management of fungal infections, which are increasing in frequency given the ever-enlarging immunocompromised patient population, largely driven by widespread use of immune-suppressive and immune-modulatory therapies (Harpaz et al., 2016). Rapid and accurate identification of fungal pathogens is known to impact on the appropriate antifungal drug choice, since fungal pathogens exhibit different antifungal susceptibility profiles (Albataineh et al., 2016; Perlin et al., 2017).

Non-culture-based technologies have been developed to aid clinical laboratories in the rapid and accurate identification of invasive fungi. These technologies include antibody detection, antigen detection of fungal proteins and polysaccharides, proteomic profiling and genomic identification by PCR and DNA sequencing (Zhang, 2013; Albataineh et al., 2016; Perlin and Wiederhold, 2017). Each technology varies in sensitivity, breadth of coverage, rapidity and ease of use. Technologies such as PCR provide rapid and sensitive detections of specific targets e.g. *Aspergillus* species directly from clinical specimens (White et al., 2015), but remain limited due to inability to detect the breadth of potentially invasive fungi. PCR/ESI-MS is a technology that combines the rapidity and sensitivity of PCR with the breadth of database coverage of more than 200 fungal species.

The PCR/ESI-MS technology had previously been evaluated for the detection and identification of fungi in both pure culture isolates and retrospectively archived frozen respiratory specimens, and were shown to be sensitive to diverse species representing fungi in all pathogenic clades (Massire et al., 2013; Shin et al., 2013). Unlike these previous studies, our current study determined both analytical and clinical performance of the PCR/ESI-MS assay in patient-consented

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prospectively collected BAL samples. For the analytical performance, sensitivity (or limit of detection) and specificity of the PCR/ESI-MS assay for fungal detection (that were lack in previous publications) were assessed. For the clinical performance, the PCR/ESI-MS method was applied to prospectively collected BAL specimens obtained from patients suspected of, or at high risk for, pulmonary fungal infections. In addition, an updated detection platform, capable of handling larger input volumes (5 mL vs. 1 mL), with an improved signature database and a more rapid processing time (<7 hours sample to answer), was used. Results were analyzed relative to comparator standard care of mycology laboratory testing, which included culture, biomarker, histopathology, and molecular data, along with clinical data from the same patients.

2. Materials and methods

2.1. PCR/ESI-MS

The PCR/ESI-MS assay was designed to detect and identify over 200 pathogenic fungi belonging to 93 genera. An additional 231 non-pathogenic fungi which were found to have little or no history of human pathogenicity were included in the assay database and reported under a non-specific fungal detection category. The pre-filled 16 well assay strips contained 15 primers targeted against widely conserved fungal genes in the small and large ribosomal subunits, as well as against more narrowly conserved ribosomal genes found in fungal mitochondria and the beta-tubulin gene. The distribution of this gene across the fungal divisions was shown in Supplement Fig. 1. A sixteenth primer was used for to control for extraction and amplification. These primers, PCR cycling conditions, and the extraction method have been shown in previous publications (Massire et al., 2013; Shin et al., 2013). After PCR amplification, the desalting method purifies the amplified nucleic acids, which are then loaded onto the ESI-MS module. The mass of the resulting amplicon is determined and the nucleotide base composition of this amplicon is calculated based upon the mass and the known nucleotide sequences of the PCR primers and target genome regions. Base compositions are determined for each unique amplicon in each well of the assay, and compared against a curated database. This database contains base composition signatures developed using data both gathered empirically and simulated from known sequences acquired from GenBank. A final identification is then triangulated using the information generated from each assay primer pair.

Each sample processed on the PCR/ESI-MS system included three controls introduced at various points in the process. (1) An extraction control was added to every sample, which was amplified by a stand-alone primer pair included with the assay. Failure to detect this control was indicative of an issue with sample extraction or other downstream failures. (2) An amplification control, or calibrant, was part of the assay formulation for each well of the assay. Each calibrant was a synthetic DNA construct design to be amplified by its associated primer pair and to be easily distinguishable from organism targets by the analysis algorithm. The calibrant was used both to reduce low levels of background noise produced by the PCR and to control for amplification issues in the absence of target template. (3) Peptides of known masses were introduced during the desalting and ESI-MS processes to provide a positive indication of the successful execution of the desalting and electrospray ionization steps, and to provide precise calibration points for the analysis of the spectra produced by the mass spectrometer.

2.2. Limit of detection (LOD) and analytic specificity

The analytical sensitivity of the assay was verified through low concentration testing of multiple strains of each fungus for which an LOD was established. Concentrations were determined by counting colony forming units (CFU) per milliliter. A "core" set of fungi was selected that exercised all assay primers and were part of the LOD study. The core fungi were: *Aspergillus fumigatus*, *Candida dubliniensis*, *Candida glabrata*, *Cryptococcus neoformans*, and a *Mucor* sp.

An initial LOD study was conducted using the five core fungi, utilizing sterile saline as a surrogate for clinical BAL matrix. Limits of detection were determined by processing 2-fold dilution series of five replicates each, and then confirming the lowest concentration at which all five replicates were positive for the target fungi by processing an additional 20 replicates. The confirmed LOD was the lowest concentration at which at least 19 of 20 replicates were positive for the target fungi. The limits of detection of 33 additional fungi that tested the phylogenetic range of the assay were also established using this method described above.

Analytical specificity studies were conducted to evaluate the ability of the assay to perform in the presence of interfering substances and the ability of the assay to detect and characterize multiple target organisms in a single sample. A cross-reactivity study was conducted in which multiple species of bacteria, viruses and non-target fungi at high concentrations was tested. The assay reproducibility was evaluated by testing multiple low concentration replicates of the core fungi across multiple assay lots and systems.

2.3. Standard of care laboratory testing data and clinical data from patients with bronchoalveolar lavage (BAL) samples

A total of 343 BAL samples were prospectively collected from 343 consented patients in the bronchoscopy suite at the Johns Hopkins Hospital, Baltimore, MD, USA, from July 2012 to December 2014. Inclusion: patients who had a clinical indication for bronchoscopy where BAL was being sent for microbiologic testing; Exclusion: patients who had tachycardia or hypotension at the time of bronchoscopy, or had a history of pulmonary hemorrhage syndrome, vasculitis, acute coronary syndrome, pregnancy, or were mechanically ventilated. Written informed consent was obtained for patients who were interested in participating.

Participating subjects had an additional BAL wash obtained for research purposes. Standard of care laboratory testing data and clinical data were abstracted from chart review and de-identified prior to analysis of PCR/ESI-MS. The study was approved by the Johns Hopkins Institutional Board Review (IRB#00072334).

All BAL samples were processed at the Johns Hopkins Hospital Microbiology Laboratory for the detection and identification of fungal pathogens using all standard of care reference tests including direct microscopic examination by calcofluor white staining, fungal culture, galactomannan (positive cutoff value: GMI 0.5), and direct fluorescent antibody (DFA) microscopic examination that is the only method used for the detection of *Pneumocystis jirovecii*. In parallel to the above reference standard of care tests, 5 mL of direct BAL fluid was tested by PCR-ESI/MS (IRIDICA™, Ibis Biosciences, CA, USA).

Patient chart review was conducted by two independent researchers. Data was abstracted using a standardized data collection instrument and included sex, age, underlying conditions, histopathological findings, radiological results, antimicrobial regimen, clinical outcomes, and laboratory findings (galactomannan results in serum and BAL, serum beta-D-glucan, and microscopy/culture results from tissues in addition to BALs). Patients with proven, probable, and possible fungal infections were defined by EORTC/MSG (European Organization for Research and Treatment of Cancer/Invasive Fungal Infections and the National Institute of Allergy and Infectious Diseases Mycoses Study Group) criteria (De Pauw et al., 2008), with some modification for *P. jirovecii* infection. Probable pneumocystis infection would apply for these patients with immunocompromised conditions, clinical features, and radiological evidence but negative by microscopic examination. Possible pneumocystis infection would apply for those patients with immunocompromised conditions and clinical features but no radiological and other laboratory findings.

3. Results

3.1. Limit of detection (LOD) and analytic specificity

The limit of detection for the five core fungal species as well as an additional 33 species were established (supplement Fig. 2). The LOD of these fungal species ranged from <1 CFU/mL to 320 CFU/mL with a mean LOD of 19.3 CFU/mL and a median LOD of 3.75 CFU/mL. Additional strains of each species for which an LOD was established were tested at near LOD concentrations. The fungal species included in the LOD and analytical sensitivity testing exercised the phylogenetic range and depth of the assay.

The analytical specificity of the assay was evaluated by challenging the assay with high concentrations (10^5 CFU/mL, copies/mL or TCID50/mL) of non-target microorganisms: *Haemophilus influenza*, *Chlamydophila pneumonia*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumonia*, *Moraxella catharralis*, *Mycoplasma pneumonia*, *Influenza virus*, *Parainfluenza virus*, *Herpes simplex virus*, *Adenovirus*, *Respiratory Syncitial virus, Coronavirus, Cytomegalovirus, Monocillium humicola, Beauveria bassiana, Sporobolomyces roseus, Oidiodendron echinulatum, Neurospora sitophila*. No cross-reactivity was observed.

The assay and system were also evaluated with regards to their susceptibility to interference from endogenous and exogenous substances expected to be present within BAL specimens. All substances, listed in supplement Table 1 were tested in the presence of near LOD levels of the core fungi. No interference or spurious detections were observed.

To evaluate the ability of the assay to correctly detect and identify target fungi in the presence of multiple targets, each of the core fungi was combined with one other member of the core set at near (3x) LOD concentrations in triplicate. All components of each mixture combination were detected and no spurious detections were observed. Additionally, a 100% detection rate was observed in reproducibility studies conducted using 180 replicates of the core fungi at near LOD concentrations, tested using multiple systems and assay lots.

3.2. Detection and identification of fungal pathogens in bronchoalveolar lavage (BAL) samples

A total of 343 patients were enrolled in this study and 343 non-duplicated BAL fluid specimens were collected over a two-year study period. Two hundred and sixty-two of these samples were included in this study. The 81 BALs were excluded due to the following reasons: 59 were due to insufficient sample volume (<5 mL); 18 were due to invalid test results in PCR/ESI-MS analysis; 4 samples were not labeled.

Ninety-two percent of tested samples (239/262) were from patients known to be in an immunocompromised state. There were 125 specimens from patients with transplantation (47.7%, 125/262), 75 from patients with malignancy (28.6%, 75/262). The detailed underlying disease spectrum of these patients are listed in the supplementary tables 2–5. Twenty-three samples were obtained from immunocompetent patients. Eighteen of these samples were negative by both reference standard assays and PCR/ESI-MS. Three samples were positive for yeast or *Candida albicans* by both methods but none of these patients had clinical signs of fungal infection, most likely suggestive of colonization. One specimen was culture positive for *Histoplasmosis capsulatum* but did not yield a detection by PCR/ESI-MS. One specimen was detected by PCR/ESI-MS as *Coccidioides immitis* but found to be culture negative.

The positive detection rate for reference methods was 34.0% (89/262), and for PCR/ESI-MS was 51.5% (135/262) (Table 1). We also assessed concordance on the genus and species levels for the 65 results which were positive by both methods, finding a genus level concordance of 70.7% (46/65) and a species level concordance of 30.1% (20/65). The overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and positive likelihood ratio + (LR+) for broad level of fungal detection by PCR/ESI-MS were 73.0%, 59.5%, 48.2%, 81.1%, and 1.8, respectively (Table 1).

Table 1
Comparison of the detection results between reference standards and PCR/ESI-MS.

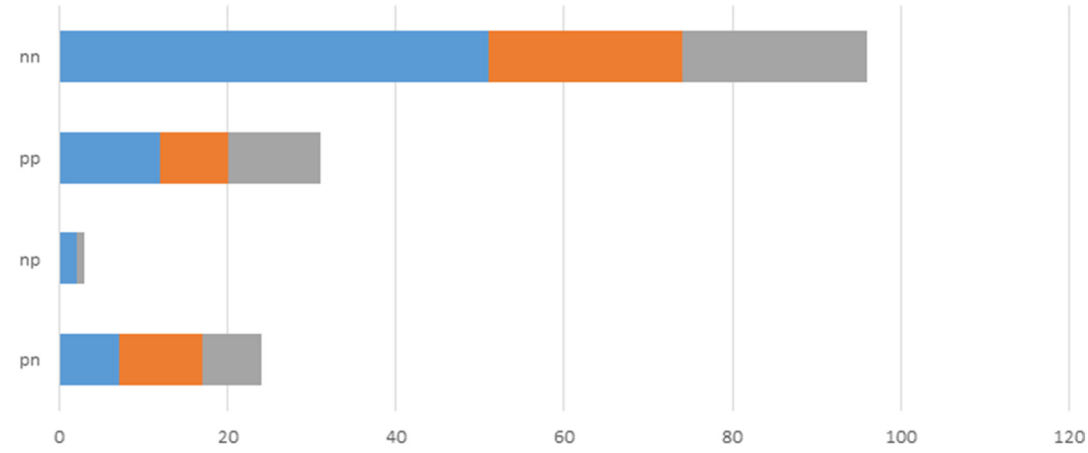
		Reference methods		Total
		Positive	Negative	
PCR/ESI-MS	Positive	65	70	135
	Negative	24	103	127
		89	173	262
Sensitivity	Specificity	PPV	NPV	LR (+)
73.0% (95%CI: 63.0–81.1%)	59.5% (95%CI: 52.1–66.6%)	48.2% (95%CI: 39.9–56.5%)	81.1% (95%CI: 73.4–86.9%)	1.8

PPV: positive predictive value; NPV: negative predictive value; LR: likelihood ratio.

We then performed a structured chart-review of the 262 patients which included clinical presentations, underlying conditions, imaging studies, and other laboratory markers. We found that 146 specimens were from patients with probable or possible fungal infections in line with the factors illustrated in EORTC/MSG criteria (De Pauw et al., 2008). Seventy-two of these specimens were from patients with transplantation (49.3%, 72/146); 31 from patients with malignancy (21.2%, 31/146), and the remaining specimens were from patients with HIV, interstitial lung disease, chronic obstructive pulmonary disease, et al (28.1%, 41/146) (Fig. 1). The most common comorbidity was lung transplantation followed by hematological malignancy (Fig. 2). Detailed abstracted clinical data from the 146 patient is listed in supplement Table 2–5. In the 146 specimens from patients with a clinical context relevant to fungal infections, the sensitivity, specificity, PPV, NPV and positive LR of PCR/ESI-MS were 90.9%, 82.3%, 60.0%, 96.9%, and 5.1, respectively (Table 2).

Aspergillus was detected in 35 BAL samples by culture and/or PCR/ESI-MS, 20 of which were considered to be related to lung infection by clinical context including underlying conditions, clinical features, and lung CT findings (Fig. 3). Eighteen of them were detected by PCR/ESI-MS; of which fifteen were verified by culture, and of which ten matched culture-based identification at the species level. Thus, samples positive by both PCR/ESI-MS and reference methods accounted for the majority of *Aspergillus* infection-related cases (75%, 15/20). Increased galactomannan values were detected in nine of these 20 *Aspergillus* infection-related specimens (ranging from 0.73 to 19.01), all of which were positive by both PCR/ESI-MS and culture-based methods. Of the 20 clinically relevant *Aspergillus* detections, none of the patients who were culture-positive had been given an antifungal agent before BAL specimens were collected, while the 3 patients who were positive by PCR/ESI-MS but negative by culture had been given an azole before the specimen was taken. As to the two cases of *Aspergillus* infection-related that were positive by culture but negative by PCR/ESI-MS, one case presented with a positive galactomannan index (GMI 1.02). This patient suffered from lung cancer and underwent chemotherapy and radiotherapy. Imaging showed tiny foci of cavitation within the anterior aspect of consolidation and some multifocal patchy foci of ground glass in both lungs, particularly in the middle and lower lung zones. The other case was a patient with idiopathic pulmonary fibrosis and left lung transplantation who was on intermittent voriconazole for months without any improvement. The culture grew *Aspergillus fumigatus* and was positive for galactomannan (GMI 0.79). This patient had a mild fever with other serious respiratory symptoms and worsening lung function for several weeks.

There were 14 *P. jirovecii* detections by PCR/ESI-MS, 7 of which were concordant with the reference DFA results (Table 3). Thirteen patients were considered to have probable pneumocystis pneumonia according to their clinical presentations, which included fever, cough, worsening pulmonary function, and imaging alterations such as ground-glass lung infiltrates. The underlying conditions of the 14 patients who had



	pn	np	pp	nn
transplantation	7	2	12	51
malignancy	10	0	8	23
other diseases	7	1	11	22

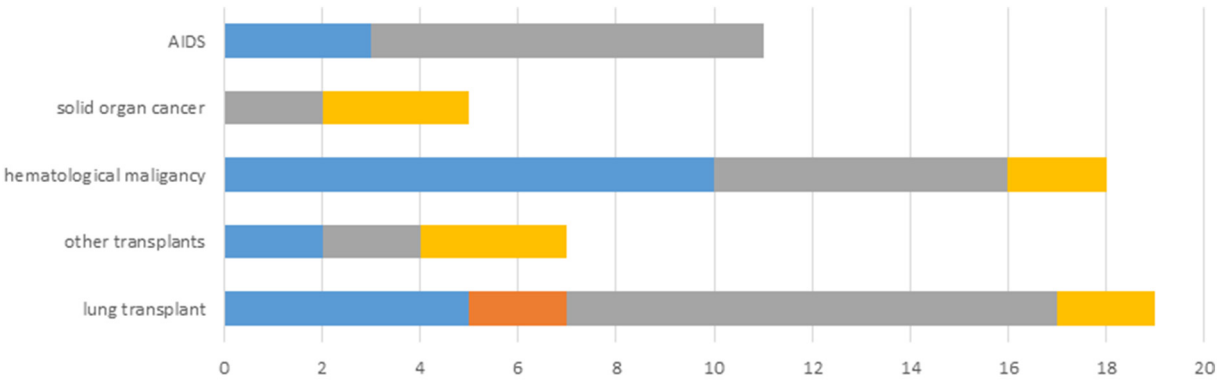
Fig. 1. Disease spectrum distribution among 146 specimens. nn: no detection from both methods; notably, 1 patient developed lymphoma post bone marrow transplant, 1 patient developed acute myelocytic leukemia post bone marrow transplant, and 1 patient developed testicular cancer with acute lymphoblastic leukemia post bone marrow transplant. pp = dual positive by reference and PCR/ESI-MS methods; notably, 1 patient developed lymphoma post bone marrow transplant. np = negative result by PCR/ESI-MS while positive by reference. pn = positive result by PCR/ESI-MS while negative by reference; notably, 1 patient developed chronic myelomonocytic leukemia post bone marrow transplant, 1 patient developed lymphoma post bone marrow transplant, and 1 patient developed HCV cirrhosis and AIDS.

positive detection of *P. jirovecii* by PCR/ESI-MS included HIV, hematological malignancy and liver transplant. The 7 patients also positive by DFA were HIV cases.

Fourteen BALs were detected positive for *Malassezia* sp. by PCR/ESI-MS but none by culture. Clinical evidence of possible fungal infection was found in two of these cases. One case was a 62 years old male with lung transplantation and imaging showing patchy ground glass. The other case was a 27 years old male with kidney transplantation and imaging showed bilateral consolidation with pleural effusions.

4. Discussion

Analytical studies of the updated PCR/ESI-MS platform demonstrated that the system and assay can detect a diverse array of fungal pathogens responsible for invasive fungal infections at low concentrations in a sterile saline matrix. One limitation was that the LOD results generated in sterile saline matrix may not be the same as if they were done in clinical BAL matrix. The system and assay were shown to provide reproducible, robust results in the presence of substances expected to be encountered in BAL specimens, and did not generate unexpected



	lung transplant	other transplants	hematological maligancy	solid organ cancer	AIDS
pn	5	2	10	0	3
np	2	0	0	0	0
pp	10	2	6	2	8
nn	2	3	2	3	0

Fig. 2. Comorbidities among patients with suspicion of IFD. pn = positive result by PCR/ESI-MS while negative by reference; np = negative result by PCR/ESI-MS while positive by reference; pp = dual positive by reference and PCR/ESI-MS methods; nn = no detection from both methods.

Table 2
Comparison of the detection results between reference methods and PCR/ESI-MS in these patients with proven, probable or possible invasive fungal infections.

		Reference methods		Total
		Positive	Negative	
PCR/ESI-MS	Positive	30	20	50
	Negative	3	93	96
		33	113	146
Sensitivity	Specificity	PPV	NPV	LR (+)
90.9% (95%CI: 76.4–96.9%)	82.3% (95%CI: 74.2–88.2%)	60.0% (95%CI: 46.2–72.3%)	96.9% (95%CI: 91.2–98.9%)	5.1

PPV: positive predictive value; NPV: negative predictive value; LR: likelihood ratio.

Table 3
Underlying condition of patients who were positive for *Pneumocystis jirovecii* by DFA and/or PCR/ESI-MS.

	PCR/ESI-MS	DFA
HIV	9	7
Lymphoma	2	0
Leukemia	1	0
Liver transplant	1	0
ILD with immunosuppressant	1	0
	14	7

ILD = interstitial lung disease; DFA = direct fluorescence assay.

detections or misidentifications in the presence of common bacterial and viral respiratory pathogens as well as these fungi not targeted in the assay, or when presented with specimens containing mixtures of low concentrations of fungi identified by the assay.

To investigate the clinical performance of the system and assay, we evaluated 262 BAL specimens from 262 patients for pulmonary fungal infection using PCR/ESI-MS, and compared findings with stand of care reference tests. Opportunistic fungal infections involve ubiquitous fungi and occur predominantly in individuals whose immune systems are compromised. In this study, 92% of the subjects were considered immune-deficient due to solid organ transplantation, bone marrow transplantation, neutropenia, chemotherapy and/or radiotherapy, AIDS, other chronic debilitating diseases, long-term usage of corticosteroids and/or use of immunosuppressants or cytokine antagonist medications. Practically, it is essential to evaluate the diagnostic accuracy of microbiological methods on the basis of clinical relevance. A structured chart review was conducted for the 262 specimens, of which 146 specimens were from patients with probable and possible fungal infections based on the revised EORTC/MSG criteria (De Pauw et al., 2008). We found that the sensitivity, specificity, PPV, NPV, and positive likelihood ratio of PCR/ESI-MS were significantly increased when compared to reference laboratory methods alone. All of the 146 patients were in an immunocompromised state, including transplant, hematological malignancy and AIDS patients who were the most vulnerable population at a high risk of invasive fungal diseases.

Although no fungal infections were found among those patients without predisposing immunocompromising factors, two fungal pathogens were detected in two patients. One strain of *Histoplasma capsulatum* was isolated by culture but not detected by PCR-ESI/MS (hence false negative) from a patient with multiple sclerosis and anemia, and imaging results showed a consolidation pattern and a 2.7 cm nodule in the right upper lobe, compatible with chronic histoplasmosis. One strain of *Coccidioides immitis* was detected by PCR/ESI-MS in a culture negative BAL. The result was indeterminate since the 70-year-old male patient did present with cough, and the chest X-ray showed some filtrates as well as mediastinal adenopathy but lacked other lab results to support coccidioidomycosis infection and a history of travel to endemic areas was not available. He was diagnosed as unspecified ocular disease, and this pathogen could have been the causative agent for this patient's eye problems (Vasconcelos-Santos et al., 2010).

Aspergillus sp. represent some of the leading causative agents of opportunistic fungal lung infection among immunocompromised patients (Baughman, 1999; Cadena et al., 2016). *Aspergillus* sp. was found to be a frequent colonizing pathogen in the airway in these populations. The development of invasive pulmonary aspergillosis depends on the level of the host immune deficiency (Patterson et al., 2016). Approximately 57% (20/35) of the isolated *Aspergillus* cases were judged to be related to pulmonary infections. In the 18 cases of probable pulmonary aspergillosis detected by PCR/ESI/MS, 83% (15/18) were concordant with culture, and half of these cases had increased galactomannan values. The PCR/ESI-MS system is rapid (6–8 hours) in comparison with culture-based methods, and can be performed directly on uncultured BAL specimens, enabling earlier targeted therapy. In this study, there was no administration of antifungals before bronchoscopy for clinically relevant specimens which were culture-positive for *Aspergillus*, while voriconazole, posaconazole, or amphotericin B had been used for those *Aspergillus* detections by PCR/ESI-MS, indicating the potential

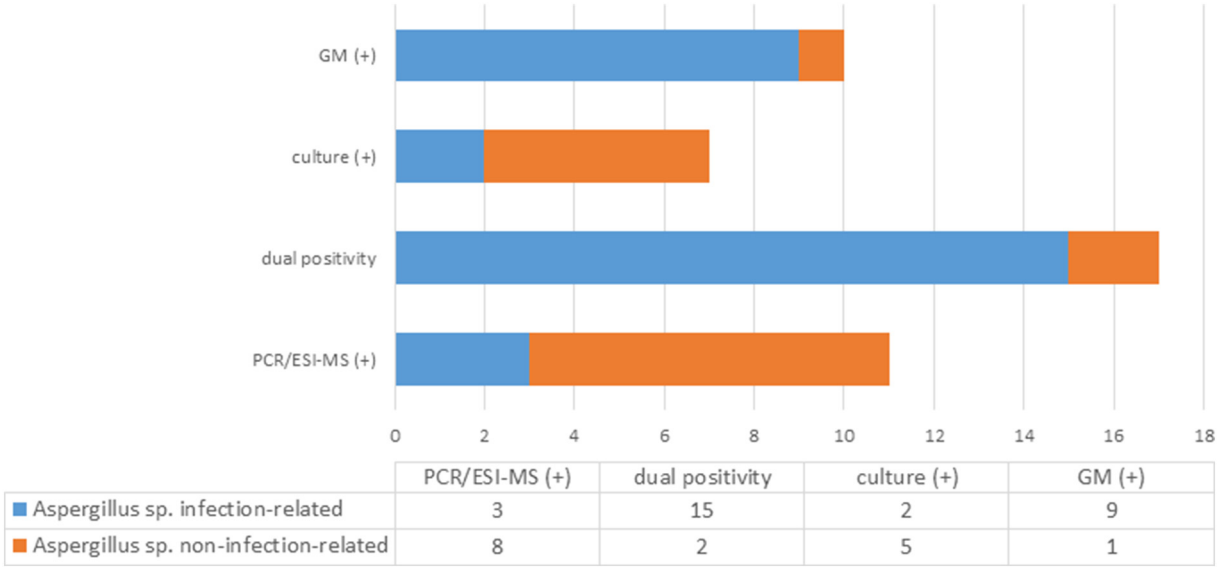


Fig. 3. The clinical relevance of *Aspergillus* sp. detected by galactomannan, PCR/ESI-MS and culture.

advantage of this approach in cases where prophylactic antifungal treatment has been initiated prior to specimen acquisition. Although PCR methods potentially have high sensitivity, there were two probable aspergillosis cases missed by PCR/ESI-MS, which could be due to poor quality of the specimens resulting in low yield of *Aspergillus* DNA (Alanio and Bretagne, 2014).

P. jirovecii remains one of the most common opportunistic pathogens in AIDS patients and occurs in patients with other causes of immunodeficiency (Thomas Jr. and Limper, 2004). The standard reference method for diagnosing *P. jirovecii* is a direct fluorescence microscopy assay on specimens obtained by BAL (Procop et al., 2004; Choe et al., 2014). There were 7 additional *P. jirovecii* detections by PCR/ESI-MS that were negative by DFA. We didn't confirm these cases by another molecular assay. However, all these patients were immunocompromised patients (Table 3) and all except one had clinical presentations and radiological evidence to support the clinical diagnosis of the disease. AIDS patients often have a high-burden of *P. jirovecii*, with a previous study showing DFA assay positive rate as high as to 55% (Choe et al., 2014). The detection rate by DFA in this study in the nine clinical-suspicious-PCP-infection HIV patients was 77.8% (7/9), while by PCR/ESI-MS was 100% (9/9). In the five non-HIV patients, detection of *Pneumocystis* was negative by DFA but positive by PCR/ESI-MS (Table 3). Patients whose immunocompromised state is not due to AIDS usually present a low burden of *P. jirovecii* and are difficult to diagnose microscopically (Seah et al., 2012; Fauchier et al., 2016). Our results showed positive *P. jirovecii* detection by PCR/ESR-MS only in non-AIDS immunocompromised patients. This finding was consistent with previous studies demonstrating that real-time PCR assays increased sensitivity to detect *P. jirovecii* infection (Hauser et al., 2011). Therefore, PCR/ESI-MS could serve as a more rapid, sensitive and specific technology for the detection of *P. jirovecii*, especially in non-AIDS immunocompromised patients. Certainly, more cases are needed for conclusive assessment. One case detected by PCR/ESI-MS was thought to be free of PCP pulmonary infection due to lack of corresponding clinical presentations and radiological evidence for the infection. *P. jirovecii* can be colonized in both HIV and non-HIV immunocompromised patients (Frealle et al., 2017). Although there are no definitive evidence suggesting the risk of reactivation and evolution of the colonization, the progression to pneumonia may need to be evaluated in cases of long-time usage of specific drugs such as cytokine blockers (Morris and Norris, 2012). The fungal biomarker β-D-glucan could be an additional useful test with high negative predictive value for the diagnosis of the disease and prognostic value for monitoring the patient treatment (Onishi et al., 2012; Damiani et al., 2013; Kutty et al., 2016).

Malassezia sp. usually colonize the skin and occasionally colonize the respiratory tract. Growth of *Malassezia* requires specific fungal culture media, which is most likely to be the reason for the universally negative culture results for the organisms detected by PCR/ESI-MS. Recently, *Malassezia* fungemia, peritonitis, arthritis and pulmonary infection have been reported in immunocompromised patients including neonates, diabetics with dialysis, and patients recovering from stem cell or solid organ transplantation (Tragiannidis et al., 2010; Velegraki et al., 2015; Pedrosa et al., 2018). Patients on total parenteral nutrition are thought to at risk of this infection (Baker et al., 2016). Although the two patients yielded clinically relevant data to support possible *Malassezia* infection detected by PCR/ESI-MS in this study, the definitive diagnosis of *Malassezia* infection in these 2 cases needs to be confirmed by another molecular method. Both patients were in a severely immunocompromised state, and lived only short periods of time following collection of the specimens studied here. PCR/ESI-MS offered a way to detect this potential pathogen in an immunocompromised population.

5. Conclusion

The overall sensitivity and specificity of the PCR-ESI/MS was found to be 90.9% and 82.3% (respectively), with a positive likelihood ratio of 5.1 in those patients who were categorized as having proven, probable or possible invasive fungal infection per the EORTC-MSG guidelines. This demonstrates the clinical utility of the technology, especially when applied judiciously within the appropriate treatment context. Additionally, in a clinical setting these results would be provided to the treating clinician within hours, as opposed to the days to weeks that can be required for the results generated from culture based methods. The range of fungi which can be detected by the technology also reduces the testing burden required for a given patient, as current molecular technologies can generally only detect a limited number of fungi. Furthermore, the automated molecular identification provided by the system does not require specialized training and knowledge required for microscopic identification of fungi from culture or histopathological specimens. It has been noted that improving the ease and rapidity of fungal diagnosis will have a positive effect on patient outcomes and hospital costs (Arvanitis et al., 2014).

PCR-ESI/MS is not the only broad spectrum molecular technology that is under development for the diagnosis of invasive fungal infections. Multiplex PCR and DNA sequencing analysis of patient specimens are among the technologies that can provide a utility similar to that which has been demonstrated here for PCR-ESI/MS. The broadening of the diagnostic toolkit available to clinicians, and the accompanying gains in speed and accuracy of diagnosis, could help to improve patient outcomes, reduce hospital costs and help combat the spread of antibiotic resistance. Unfortunately, PCR-ESI/MS was removed from the market by Abbott Laboratories in 2017 primarily due to financial consideration (Ozenci et al., 2018). However, our present data, as well as a recent publication using PCR-ESI/MS to improve fungal diagnosis in hematological patients (Krifors et al., 2019), sheds new light on the importance of developing these non-culture based broad panel molecular assays to enhance the detection of fungal pathogens in those patients with invasive fungal infections. The continuing development and clinical investigation of these technologies should be pursued.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2020.114988.

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