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The Performance of Quantitative Real-Time Polymerase Chain Reaction and Galactomannan for Invasive Fungal Infections in Patients with Hematologic Malignancies

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Abstract

Background: Non-invasive methods to diagnose invasive fungal infections are needed to improve the poor outcome of these infections. We conducted this study to evaluate the diagnostic values of quantitative real time polymerase chain reaction and galactomannan in hematological malignancy patients.

Results: Eighty-two consecutive patients with cancer were identified and prospectively followed for three months. Molecular testing using a quantitative polymerase chain reaction assay amplifying two primers 5.8S and 18S rRNA fungal genes and galactomannan was performed on 1540 blood samples and correlated with clinical data. Amplification of the 5.8S rRNA fungal gene had significantly higher sensitivity than amplification of the 18S rRNA gene for samples from either source (for blood samples: 90% vs 50%, p=0.007; for serum samples: 64% vs 5%, p<0.001).

Conclusions: Galactomannan when used alone had a sensitivity of 38% and a specificity of 100%. The sensitivity of the combination assay of quantitative polymerase chain reaction plus galactomannan was significantly higher than that of GM test alone (71% vs 38%, p=0.03). Detection of the 5.8S rRNA fungal gene had significantly higher sensitivity than the 18S rRNA gene for samples from either source blood or serum. The combination of quantitative polymerase chain reaction plus galactomannan assay improved the diagnostic value of invasive fungal infections.

Keywords: Hematologic malignancies; Invasive pulmonary fungal infections; Galactomannan; Real-Time polymerase chain reaction

Introduction

Invasive fungal infections (IFIs) remain a major cause of morbidity and mortality in immunocompromised patients, ranging between 60%-90%, especially in those patients with hematological malignancies who receive induction chemotherapy or undergo hematopoietic stem cell transplantation (HSCT) [1,2]. The critical challenge is achieving an accurate and early diagnosis of IFI and selecting the appropriate antifungal therapy in order to improve the outcome of patients with this life-threatening infection. The absence of reliable, rapid, non-invasive and cost effective diagnostic procedures remains a major setback in the successful management of IFIs. Early diagnosis of invasive aspergillosis (IA) appears to be associated with an increased survival rate and better outcome [3,4]. The gold standard for definitive diagnosis requires deep tissue biopsy to obtain culture and tissue specimens for histopathology. However, this invasive procedure may not be feasible in the setting of thrombocytopenia or the critical condition of the patient. In addition, the clinical signs of infection are often nonspecific and conventional tests, such as blood culture or bronchoscopy, show very low sensitivity [5]. A systematic use of chest CT is a useful tool for diagnosis and outcome evaluation of invasive pulmonary aspergillosis [3]. Therefore, a novel noninvasive method for the diagnosis of IFIs is highly needed. Moreover, among the non-invasive molecular methods, galactomannan (GM) appears to be the most studied diagnostic marker [6,7]followed by 1-3 β-D-glucan [8] and quantitative real-time PCR (qPCR) [9-11]. All of these methods offer a promising alternative to conventional methods by reducing the time for identification and improving sensitivity. Few prospective studies of qPCR in comparison and in combination with GM have been performed in patients with HM. We, therefore, conducted this study to determine the diagnostic value of qPCR and GM in cancer patients.

Methods

Study population

Between October 2002 and September 2005, we prospectively evaluated all patients at high risk of developing IFIs, including patients undergoing stem cell transplantation or receiving intensive chemotherapy for HM. Eighty-two patients were evaluable for the study and were followed for three months. Patients were divided into the following groups: 22 patients with IA (proven or probable) , chosen according to the criteria developed by the consensus of the European Organization of the Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) [6]; 17 patients with other mold infections, such as Fusarium, zygomycosis, and Scedosporium; and 23 patients with candidemia. All these diagnoses were apparent upon enrollments into the study. In addition, we selected 20 control non-neutropenic patients with solid tumors and without any radiological or clinical evidence of IA or risk factors for IFI. A total of eight blood draws were obtained from each patient, two collected on week one and one sample every other week for 12 weeks of follow up. Samples were collected prospectively

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and patients were assessed and treated for their fungal infections. Patient demographics and clinical characteristics including age, underlying disease, type of transplantation, steroid use, neutropenia, and antifungal therapy used during the study period were recorded.

This study was approved by the University of Texas M.D. Anderson Cancer Center Institutional Review Board and a waiver of informed consent was obtained for this study.

Fungal DNA extraction from blood and serum samples

Extraction of DNA from 1.5-3mL of whole blood was performed according to manufacturer's protocol using the Gentra PURGENE auto-extraction unit (Qiagen, Valencia, CA) and eluted in 200µL of nuclease free TE buffer yielding an average concentration of 300µg/mL. For serum samples DNA was extracted according to manufacturer's protocol from 1-2mL samples using a silica/guanidinium isothiocynate extraction with the NucliSENS automated extraction platform (Biomerieux, Durham, NC). Both Gentra PURGENE and NucliSENS auto extractor are extracted in a contained system thus making the extraction process more user friendly and decreasing the chance for DNA contamination from the user.

Real-time PCR for amplification and detection of Aspergillus DNA

Real-time PCR assays for fungal DNA amplification were performed with the Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). For the 5.8S rRNA gene amplification fungal DNA for a genomic standard was extracted from Aspergillus fumigatus (AF293) using UltraClean Microbial Isolation Kit (MO BIO Inc, Carlsbad, CA). Subsequently, results from the genomic based standard were converted to copies of the 18S rRNA gene per mL of blood or serum based on published literature [12]. For the 18S rRNA gene a copy based standard ranging from $1x10^1 - x10^8$ copies/well was used to generate the standard curve (10). Finally, commercially available human genomic DNA (ABI, Foster City, CA) was used as quality control to indicate success of the amplification reaction.

The sequence of the PCR primers and probes were based on the sequences of the 18S and 5.8s rRNA genes conserved for A. fumigatus previously submitted to the GenBank database. Primer sequences for the amplification of the 18S rRNA gene [13] and 5.8S rRNA gene [12] have been previously published [13]. For the quantitative detection each primer (5.8S and 18S) was labeled on the 5' end with FAM (6-carboxy-fluorescein) and TAMRA (6-carboxy-teremethyl-rhodamine) as a quencher at the 3' end. A 50 µL total reaction volume contained 10uL of DNA purified from whole blood or serum, 40µMol if each forward or reverse primer, 5µMol FAM/TAMRA fluorescent probe, and 25 µL ABI 2x Universal master mix containing 400 µmol/L dNTPs, 5mmol/L magnesium chloride, and 3U TaqGold polymerase (ABI). Each qPCR assay (5.8S and 18S) was performed independently. Samples were run in triplicate with duplicates of the sample being probed for the 5.8S or 18S rRNA fungal genes and the third well multiplexed for the fungal genes as well as an exogenous control. The master mix for this control assay is as above with the addition of 20x 18S rRNA mgbVIC probe (ABI).

Galactomannan assay

The quantity of GM in each sample was measured by using the Platelia Aspergillus EIA test kit (Bio-Rad). Coded serum samples were thawed and analyzed in batches, as directed by the manufacturer. Optical densities were read at 450 nm - 620 nm. Results were recorded as an index relative to the mean optical density of the threshold controls. Samples that had index values >0.5 were considered positive and underwent repeated testing to ensure positive results. Evaluable patients were required to have two independent serum samples available for analysis.

Definitions

Neutropenia was defined as an ANC <500 cells/ml. Proven IA was defined as clinical signs and symptoms compatible with aspergillosis and radiological findings highly suggestive of IFIs in the presence of the proper host factors with histopathologic or microbiologic documentation of disease from biopsied tissue samples.

Probable IA was defined as clinical signs and symptoms and radiological findings that were highly suggestive of IFIs plus a positive culture from bronchoalveolar lavage fluid or sputum samples in patients with hematologic malignancy with appropriate host factors.

Proven and probable other mold IFIs was defined according to EORTC/MSG criteria.

Candidemia was defined as the isolation of Candida species from blood cultures in the presence of signs and symptom of systemic infection. PCR was considered positive if one sample tested positive. Galactomannan was considered positive if two consecutive samples tested with GM index ≥ 0.5.

Statistical methods

The sensitivity, specificity, and predictive values of diagnostic tests were calculated according to their definitions. Sensitivity and specificity comparisons were performed by chi-square or Fisher's exact tests, as appropriate. The statistical significance was set at $p \leq 0.05$. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

Results

The analysis included 82 patients. All patients in the mold infection group had hematological malignancy as their underlying disease and all received antifungal therapy. Approximately 54% had received HSCT. Seventy-two percent received steroids and 79% received immunotherapy. That includes granulocyte macrophages colony – stimulating factor and gamma-interferon therapy. In the candidemia group, 91% of the patients received antifungal therapy and 43% received immunotherapy (Table 1).

Characteristic	Aspergillosis	Other mold	Candida	Negative control
	(n = 22) n (%)	(n = 17) n (%)	(n =23) n (%)	(n = 20) n (%)
Age (years), median (range)	60 (10-78)	51 (22-73)	55 (23-81)	53 (18-71)
Gender				
Male	16 (73)	9 (53)	12 (52)	10 (50.0)
Female	6 (27)	8 (47)	11 (48)	10 (50.0)
Underlying disease				
Hematological	22 (100)	17 (100)	11 (48)	0 (0)
Solid tumor	0 (0)	0 (0)	12 (52)	20 (100)
HSCT	12 (55)	9 (53)	2 (9)	0 (0)
Antifungal therapy prior to PCR	22 (100)	17 (100)	21 (91)	2 (10)
Neutropenia at infection onset	5 (23)	6 (35)	6 (26)	1 (5)
Use of steroids	17 (77)	11 (65)	9 (39)	11 (55)
Immunotherapy	18 (82)	3 (76)	10 (43)	14 (70)

HSCT; hematopoietic stem cell transplantation; PCR; polymerase chain reaction

Table 1: Demographic characteristics of different infection patients and control patients.

A total of 794 valid whole blood samples as well as 746 serum samples were tested by PCR.

Sensitivity, specificity, PPV and NPV for PCR assays amplifying the 18S rRNA gene and 5.8S rRNA gene from serum and whole blood for different groups of IFIs (including patients with IA, patients with other mold infections ,and patients with candidemia) (Tables 2-4). Furthermore, the value of GM in conjunction with PCR was assessed for its impact on the diagnostic yield for IA.

For PCR diagnosis on patients with aspergillosis, amplifying the 5.8S rRNA gene had significantly higher sensitivity amplifying the 18S rRNA gene for samples from either source (for blood samples: 90% vs 50%, p=0.007; for serum samples: 64% vs 5%, p<0.001). Results from the 5.8S rRNA assay show that blood samples tended to have significantly higher sensitivity than serum samples (90% vs 64%, p=0.07) (Table 2).

Sample source	rRNA Gene	Sensitivity	Specificity	PPV	NPV
		(%)	(%)	(%)	(%)
Blood	18s	50	80	73	59
Blood	5.8s	90	40	61	80
Serum	18s	5	84	25	46
Serum	5.8s	50	50	52	48

Note: The controls are negative controls (patients without proven probable or possible fungal infections).
PCR; polymerase chain reaction; PPV; positive predictive value; NPV; negative predictive value

Table 2: Performance of PCR test in patients with invasive aspergillosis.

Sample source	rRNA Gene	Sensitivity	Specificity	PPV	NPV
		(%)	(%)	(%)	(%)
Blood	18s	35	80	60	59
Blood	5.8s	71	40	50	62
Serum	18s	13	84	40	53
Serum	5.8s	40	50	23	43

PCR; polymerase chain reaction; PPV; positive predictive value; NPV; negative predictive value

Table 3: Performance of PCR test in patients with other mold infection.

Sample source	rRNA Gene	Sensitivity	Specificity	PPV	NPV
		(%)	(%)	(%)	(%)
Blood	18s	17	80	50	46
Blood	5.8s	61	40	54	47
Serum	18s	5	84	25	43
Serum	5.8s	13	50	23	33

PCR; polymerase chain reaction; PPV; positive predictive value; NPV; negative predictive value

Table 4: Performance of PCR test in patients with candidemia using two primers 18.s and 5.8s .

Diagnosis test	Sensitivity	Specificity	PPV	NPV
	(%)	(%)	(%)	(%)
18R rRNA qPCR assay -Blood	50	80	73	59
GM-EIA	38	100	100	61
Combination (PCR 18s + GM-EIA)	71	80	79	73
5.8S rRNA qPCR assay -Blood	90	40	61	80
GM-EIA	38	100	100	61
Combination (PCR 5.8s + GM-EIA)	95	40	63	89

PCR; polymerase chain reaction; PPV; positive predictive value; NPV; negative predictive value; qPCR, quantitative real time PCR; GM, galactomannan

Table 5: Performance of the combination of galactomannan test and PCR tests with primers rDNA 18s and rDNA 5.8s in the diagnosis of IA.

For negative control patients, the 5.8S rRNA assay had significantly lower specificity than the 18S rRNA assay for samples from either source (for blood samples: 20% vs 80%, p<0.001; for serum samples: 50% vs 84%, p=0.04). There was no significant difference in specificity between blood samples and serum samples (80% vs 84%, p> 0.99) for the 18S rRNA assay.

For PCR diagnosis in those with other mold infections, the sensitivity ranged from 13 (when using 18s rDNA primer for serum samples) to 71 (when using 5.8s rDNA primer for blood samples) (Table 3). Regarding the patients with candidemia, the sensitivity of the PCR tests ranged from 5 (when using 18s rDNA primer for serum samples) to 61 (when using 5.8s rDNA primer for blood samples) (Table 4).

There were 21 patients with aspergillosis and 20 negative controls (solid tumor) that were evaluable for the 18S rRNA qPCR and GM-EIA combination assay. Sensitivity, specificity, PPV and NPV are shown for each independent assay and the combination assays are shown in Table 5.

The sensitivity of combination assay was significantly higher than that of GM-EIA alone (71% vs 38%, p=0.03), but not significantly higher than that of 18S qPCR alone (71% vs 50%, p=0.15). The specificity of the combination test was the same as that of 18S qPCR alone (80%), which was as expected since the specificity of GM-EIA test is 100%.

Discussion

We evaluated the utility of qPCR assessing two different conserved fungal genes in whole blood and serum samples as well as the values of GM as adjunct detection methods for the diagnosis of IFI in high-risk patients with hematologic malignancy. We found that 5.8S qPCR assay was associated with better diagnostic yield regardless of the source (blood or serum) and the sensitivity was significantly improved when combined with GM.

Several studies have highlighted the advantage of using PCR technology in a variety of clinical specimens (i.e., blood, serum, or bronchoalveolar lavage [BAL]) and targeted different ribosomal RNA genes such as 5.8S, 18S, and 28S. However, the optimal specimen for detection of IFIs is still debatable. Some groups prefer to use serum samples [13-15], while others prefer to use whole blood [7,10,16-18]. Löefler et a1. [9] demonstrated that DNA extraction from plasma is complex and the sensitivity of plasma PCR is lower than that of the PCR performed on whole blood samples. In our study we compared whole blood and serum samples collected simultaneously from patients. Samples were collected for three independent types of fungal infections from cancer patients who, due to their disease, were at high risk of developing fungal infection. Our data showed that in patients with IA, other molds and candidemia, the performance of qPCR on whole blood samples was associated with higher sensitivity, positive and negative predictive values. Our data are consistent with previous studies by White et al. [17] suggesting that PCR performed on whole blood had better diagnostic yield than using other specimens. Furthermore, our group previously reported that PCR performed on BAL samples was associated with high specificity and negative predictive values for invasive pulmonary aspergillosis [19].

BAL samples may not be ideal since they are very prone to contamination, as spores of Aspergillus are often present in the upper

respiratory tract which may enable the test to differentiate colonization from infection [20,21]. The study by Heng et al. suggests that major clinical utility of GM and PCR in BAL would be to rule out a diagnosis if IPA if results were negative [22]. Hence, BAL may not be an appropriate choice and using tissue sample could be a better alternative. Few studies used PCR to detect fungal pathogens from biopsy specimen and tissue samples [20,23]. However, obtaining deep tissues biopsy requires an invasive procedure that might not be feasible in patients with profound thrombocytopenia or those who are critically ill.

In addition, several studies have suggested that amplifying different conserved genomic regions could be useful in improving the diagnostic yield of IFIs [24-27]. However, a consensus on which gene to amplify has yet to be reached. In our studies we showed that amplification of the 5.8S rRNA gene was preferable to the 18S rRNA gene for the detection of IFIs .These observations were validated among the three different groups of fungal infections we studied: patients with IA, those with other mold infections, and one with candidemia. In all three groups amplification of the 5.8S rRNA gene was associated with significantly higher sensitivity and negative predictive value than the 18S rRNA gene. Similarly, the sensitivity using whole blood samples was better than serum samples. White et al., in a multicenter study comparing two genes, 18S rRNA and 28S rRNA, showed that 28S was more specific than 18S [25]. Furthermore, the sensitivity, specificity, and positive predictive values as well negative predictive values were higher with the 28S gene compared to the 18S gene.

Despite addressing these challenges with sample and gene choices for the utility of qPCR in making the diagnosis of IFI, the diagnostic yield remains suboptimal, therefore, incorporating the diagnostic assay of GM to increase the diagnostic yield when used in combination with qPCR. Use of both assays in combination may permit better performance in the final diagnosis of IFI, and could improve clinical outcomes through timely initiation of antifungal therapy.

We further analyzed our data regarding the index level of serum GM and its correlation with patient outcomes. Our data showed that The GM index levels were three-fold higher among the patients who failed antifungal therapy compared to those who responded to therapy. Also, the GM index remained positive > 0.5 despite therapy in 56% of the patients who failed therapy versus 25% among the responders. Our results support that the GM values correlate with response and outcome whereby a higher GM was associated with worse outcome. Similarly, Foy et al. reported a significantly higher response rate among patients whose GM level normalized post therapy [28]. Maertens et al. [27] showed that serum GM Index outcome strongly correlates with survival and response outcome in neutropenic patients. In addition, Miceli and colleagues postulated that the serum GM Index should be considered as surrogate endpoint in clinical trials of IA and as a marker of response to therapy in patients with hematological cancer and aspergillosis [28]. Hebart et al. [9] showed that the reduction of fungal load or negative PCR was associated with good response, whereas persistence or an increase in the fungal load was correlated with failure. This study has several limitations which may affect the diagnostic yield of GM and PCR for invasive aspergillus. Furthermore, Aguado et al. suggested that PCR should be used to complement rather than replace the serum GM assay as the basis of a diagnostic-driven strategy for preventing IA [29].

Regardless of the sample type or gene amplified we chose, in our study the sensitivity, specificity, negative and positive predictive values were sub optimal for each of the qPCR and GM assays. This could be due to sub-clinical infections in some patients, the frequency of prospective sampling which could be the weakness of our study, or the use of active antifungal agents that may lower the fungal burden to a level that could not be detected by either a qPCR or GM assay alone. Another limitation is the small numbers of case patients with proven or probable invasive pulmonary aspergillosis.

Conclusions

In conclusion, we demonstrated that the selection of primer 5.8S rRNA gene and whole blood samples yielded better diagnostic values than the 18S rRNA gene and/or serum samples, in this patient population. Furthermore, when the PCR tests were done simultaneously with GM assay, this combination appeared to be a useful tool in improving the diagnostic yield of IFI. Therefore, consideration should be given to the simultaneous use of these tests as an IFI molecular diagnostic panel. This may lead to the early diagnosis of fungal infection and initiation of appropriate antifungal therapy that will result in improving the outcome of patients with this life-threatening infection.

Competing Interests

The authors declare that they have no competing interests.

Author Contributions

Ray Hachem participated in the design and writing of the study, Ruth Reitzel performed testing of galactomannan and PCR, Ying Jiang performed the statistical analysis, Jose Rivera performed testing of PCR, Roy F. Chemaly participated in the design and writing of the study, Xiang-Yang Han assisted in the testing of galactomannan, and Issam Raad participated in the design and writing of the study.

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