Clinical Medicine Insights: Oncology

SHORT REPORT

Early Diagnosis of Invasive Aspergillosis in Neutropenic Patients. Comparison between Serum Galactomannan and Polymerase Chain Reaction

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Abstract

Background: Invasive aspergillosis (IA) is a major cause of morbidity and mortality in profoundly neutropenic patients, so early diagnosis is mandatory.

Aim: Consecutive patients with hematological malignancies undergoing intensive chemotherapy were screened for IA with two different methods which were compared.

Methods: From October 2000 to August 2003 we tested 1311 serum samples from 172 consecutive patients with a polymerase chain reaction assay and between April 2005 and April 2008 we tested 806 serum samples from 169 consecutive patients with a Galactomannan (GM) test. Bronchoalveolar (BAL) samples were obtained whenever the patient's condition allowed and tested with either method.

Results: The serum PCR assay had a sensitivity of 75.0% and a specificity of 91.9% and the serum GM assay had a sensitivity of 87.5% and a specificity of 93.1%, ($P > 0.05$). The presence of two or more consecutive positive serum samples was predictive of IA for both assays. BAL GM/PCR was positive in some patients without serum positivity and in patients with 2 or more positive serum GM/PCR.

Conclusions: No significant differences between the 2 serum tests were found. The GM assay has the advantage of being standardized among several laboratories and is incorporated in the criteria established by the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycosis Study Group (EORTC/MSG), however is much more expensive. BAL GM and PCR sampling aids in IA diagnosis but needs further validation studies to differentiate between colonization and true infection in cases where serum GM or PCR are negative.

Keywords: hematological malignancies, intensive chemotherapy, neutropenia, invasive aspergillosis (IA), early diagnosis, serum PCR and Galactomannan (GM)

Clinical Medicine Insights: Oncology 2010:4 81–88

This article is available from http://www.la-press.com.

Introduction

Despite the latest therapeutically advances, IA is still a major cause of morbidity and mortality among hematological patients under intensive chemotherapy or hematopoietic stem cell transplantation (HSCT).[1,2]

Diagnosis of IA is difficult and challenging. Clinical manifestations are non specific and IA proof depends on invasive procedures that usually the patient does not tolerate.[3] Thus, antifungals have been used empirically for decades in refractory neutropenic fever with high costs and toxicity.[4–6] Non invasive methods for early diagnosis are of great value if a pre-emptive approach is to be chosen.[7,8] The detection of the GM antigen, a constituent of polysaccharide fungal cell wall and the detection of fungal DNA by a PCR method[9] have been developed with the aim to quickly screen patients for IA.

We compared the value of the two screening methods (GM and PCR) in similar patients (hematological malignancies and profoundly neutropenic) in the same conditions (same open ward with HEPA filters). Between 2000 and 2003[10] serum PCR assay was performed and from 2005 to 2008 GM serum detection was done.

Patients and Methods

Study population

From October 2000 to August 2003, 172 patients (median age 50 years) with acute myeloid leukemia (AML) (119 Patients), acute lymphoblastic leukemia (ALL) (26 patients) underwent intensive chemotherapy as induction or consolidation therapy and 27 patients received high dose chemotherapy in an autologous hematopoietic stem cell transplantation (auto-HSCT) setting. All patients were screened by PCR twice a week since admitted in the ward.

Between April 2005 and April 2008, 169 patients (median age 55 years) with AML received intensive chemotherapy (107 patients) as induction or consolidation therapy and 62 patients received high dose chemotherapy for auto-HSCT. Again all patients were screened twice a week for GM analysis.

Patients' characteristics are summarized in Table 1.

Table 1. Patients' characteristics.

	PCR	GM
N° patients	172	169
Median age/range (years)	50/16–77	55/15–75
Male/female	102/70	87/82
N° neutropenic episodes	418	321
Median neutropenia (days)	26	11
Treatment episodes		
- Induction chemoterapy	141	175
- Consolidation chemoterapy	127	59
- Reinduction chemoterapy	123	25
- Autologous HSCT	27	62

Abbreviations: HSCT, hematopoietic stem cell transplantation; PCR, polymerase chain reaction; GM, galactomannan.

Definitions

Febrile episodes were classified according to EORTC criteria as fever of unknown origin, fever with clinical documentation and fever with microbiological documentation.

Fever was defined as two or more consecutive measurements of axillary temperature ≥38 °C and neutropenia as a neutrophil count below 500/mL for at least 7 days.

Fungal infections were classified according to EORTC/MSG revised consensus:[11] Proven IA requires culture and identification i.e. histopathology or direct microscopic examination of a specimen obtained by needle aspiration or biopsy in which "hyphae" are seen accompanied by evidence of associated tissue damage and recovery of a mould by culture of a specimen obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process.

Probable IA relies on 3 elements, namely, a host factor that identifies the patients at risk (neutropenia <500/mL for >10 days, HSCT, prolonged use of corticosteroids, treatment with T-cell immunosuppressors); clinical signs and symptoms consistent with the disease entity (lower respiratory tract fungal disease with presence of 1 of the following 3 signs on CT: dense well-circumscribed lesions with or without a halo sign, air-crescent sign, cavity; and mycological criteria: direct tests (cytology, direct microscopy or culture) showing the presence of mould in sputum, BAL fluid, bronchial brush) and indirect tests (detection of antigen or cell-wall constituents, such as GM antigen in plasma, serum or BAL fluid). Cases that meet the criteria for a host factor and a clinical criterion but for which mycological criteria are absent are considered possible IA.

Study Design and Methods

Peripheral blood samples from patients were screened twice weekly for both methods since admission in the ward. If a positive value was obtained the patient would be screened everyday for three consecutive days in the first week and then twice weekly again. Blood samples were collected using 5-ml vacutainer tubes with EDTA to minimize chances of environmental contamination. The whole blood was aseptically transferred using a disposable pipette with a filtered tip into a centrifuge tube after careful removal of the vacutainer's rubber stopper. Besides that, to avoid false GM positives, blood samples were always scheduled to be drafted before antibiotics administration. As for PCR assay, in order to minimize false positives, from contamination from the environment or contamination of reagents by fungal DNA, procedures were done in a flow cytometry chamber and reagents were submitted periodically to amplification procedure with fungal specific probes as negative controls in parallel with patient's samples.

Serum and BAL PCR assay

Blood samples, BAL samples, fungal DNA extraction and PCR conditions were performed as described.[10,12] The whole process of amplification (denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 45s, 62 °C for 1 min and 30s, 72 °C for 2 min, ending in a final elongation step at 72 °C for 10 min) was done using *Taq* polymerase (Gibco BRL) and pan-fungal primers (forward 5′-ATTGGAGGGCAAGTCTGGTG-3′ and the reverse 5′-CCGATCCCTAGTCGGCATAG-3′)[13] that bind to the conserved regions of the fungal 18S rRNA gene sequence, yielding PCR- amplified products of 197 bp through a 1,8% agarose gel stained with ethidium bromide and UV photographed (Kodak 120 camera system). Established PCR negative and positive controls were used in every assay. Positive controls were obtained from DNA extracted from mould cultured in the laboratory of microbiology especially for the purpose. Negative controls were established as stated above.

Serum and BAL GM assay

An enzyme-linked immunosorbent assay-based kit is commercially available as the Platelia *Aspergillus* GM EIA (Bio-Rad Laboratories, Redmond, Wash) and was cleared by the FDA for diagnostic use in May 2003. It is an immunoenzymatic sandwich microplate assay that uses monoclonal antibodies that bind to side-chain residues of the GM molecule. A sandwich enzyme immunoassay format is used for detection. The Platelia *Aspergillus* GM EIA was used to quantify GM in accordance with the manufacturer's instructions. Briefly, after thawing, serum samples were mixed well, and 300 uL of each sample was added to 100 uL of 4% EDTA treatment solution, boiled for 3 min, and centrifuged at 10,000 g for 10 min. Supernatant was added to a reaction mixture containing conjugated anti-GM EB-A2 antibody, and the mixture was incubated in microtiter plates precoated with the same antibody (EB-A2) for 90 min at 37 °C and then incubated again with 200uL of tetramethylbenzidine solution for an additional 30 min in the dark. Reactions were stopped with 100 uL of 1.5 M sulfuric acid, and optical densities (ODs) at 450 and 620 nm were read. Positive and negative controls (provided in the kit) were included in each assay. Results were recorded as an index relative to the OD of the threshold control (GM index OD sample/OD threshold control). Both serum and BAL GM samples were considered positive when index value was $\geq$0.5 ng/mL.[14–16]

Complementary diagnostic procedures

Chest CT scans were performed early in the course of neutropenic fever refractory to antibiotics and when a positive sign (dense well-circumscribed lesions with or without a halo sign, air-crescent sign and cavity) was present, sequential CT scans were repeated until response. Bronchoscopy with BAL was performed whenever necessary and if the patient's condition allowed. A total of two 20 mL aliquots of sterile 0.9% saline was instilled into each affected bronchus and aspirated into sterile collection traps.

Antifungal treatment

No antifungal prophylaxis was given to our patients in either period.

Prompt antifungal therapy was initiated if patients met the criteria of proven, probable or possible IA as stated above. Deoxycholate amphotericin (1 mg/kg/day) or Liposomal B amphotericin (3 mg/kg/day) was used as first line therapy in the PCR

study period while Voriconazole (6 mg/kg q 12 hours intravenous on day 1 and then 4 mg/kg q 12 hours intravenous on day 2 onwards) was the antifungal of choice during the GM study period.

Statistical analysis

Sensitivity and specificity were calculated for the serum GM and the PCR assay with standard binomial probabilities with a 95% confidence interval (CIs). For the differences between both methods the *P* values were calculated by Fisher's exact test.

Results

The total number of samples of PCR and GM tested according to febrile episodes and the numbers of positive samples are shown in Table 2. The PCR screening was performed in an average of 3.14 samples per episode and in an average of 6.8 samples per patient. The GM assay was performed in an average of 2.51 samples per episode and an average of 6.76 samples per patient. In the PCR study, 60 among 151 episodes of fever with clinical documentation corresponded to pneumonia, of which 20 fulfilled the requirements for IA (2 Proven/18 Probable) according to EORTC/MSG criteria. In the GM study 40 among 121 episodes of neutropenic fever were pneumonia of which 8 had IA criteria (2 Proven/6 Probable).

All patients with 2 or more consecutively GM/PCR positive serum samples developed an IA. Patients with 2 or more positive serum GM also had a positive BAL GM test but some patients without positive serum GM had positive BAL GM. PCR was equally performed on BAL samples and matched PCR positive serum samples whenever performed. However some cases without serum PCR positivity were BAL PCR positives. A single patient with persistently negative serum GM and BAL GM had a proven IA diagnosis. Serum PCR and GM results analysis according to the final diagnosis using the EORTC/MSG criteria are detailed in Table 3.

Antifungal therapy was initiated during both study periods either in the presence of at least 2 consecutive positive PCR or GM results or in the presence of strong clinical evidence even when both PCR and GM were negative. PCR negativation and GM index gradual reduction with normalization were observed and matched CT scan response.

PCR screening predicted the appearance of IA by a median of 17 days and GM predicted by a median of 5 days. The PCR study had a sensitivity of 75% and the serum GM method had a sensitivity of 87.5% ($P = 0.64$). The specificity of both tests was 91.9% and 93% respectively as detailed in Table 4.

Discussion

IA has a high mortality rate (60%–90%) explained by the diagnostic delay due to the limitation of current diagnostic tests, so early IA diagnosis in patients undergoing intensive chemotherapy or HSCT remains a significant challenge to overcome. In the majority of cases the typical clinical signs according to EORTC/MSG criteria are non-visible and often develop too late in the course of infection.[3] Thus chest radiography is too insensitive to diagnose IA at an early stage and chest CT scan, although an important diagnostic tool that has played a pivotal role in IA diagnosis, remains unspecific, since initial nodular lesions surrounded by areas of lower attenuation "halo sign" and late cavitations following neutrophil recovery creating the "air crescent sign", may be due to other species of fungi (*Zygomycetes, Fusarium, Scedosporium*) and even bacteria (*Pseudomonas aeruginosa*)[17,18] and in several occasions these features are not present at all. Furthermore, blood cultures are not useful since they are

Table 2. Samples of GM and PCR tested according to classification of febrile episodes.

Classification	N° episodes	Tested samples	Positive tests
	PCR/GM	PCR/GM	PCR/GM
Without fever	39/13	80/28	1/3
Unknown origin	75/68	210/113	10/0
Clinical documentation	151/121	602/337	22/34
Microbiological documentation	153/119	419/328	24/17

Abbreviations: PCR, polymerase chain reaction; GM, galactomannan.

Table 3. PCR and GM results and final diagnosis according to EORTC-MSG.

PCR	With IA	Without IA	Total patients
Positive	15	14	29
Negative	5	159	164
Total patients	20	173	193
GM	**With IA**	**Without IA**	**Total patients**
Positive	7	11	18
Negative	1	150	151
Total patients	8	161	169

Abbreviations: PCR, polymerase chain reaction; IA, invasive aspergillosis.

often negative even in disseminated infection and take too long to become positive (median 15 days). Tissue biopsy and fluids obtained by invasive procedures (transthoracic percutaneous needle aspiration, video assisted thoracoscopic biopsy, bronchoscopy) considered the cornerstone of diagnosis of this entity are not always possible to obtain because the diagnostic procedure can not be performed directly in patients who are not fit (hypoxemic, hemodynamically instable and thrombocytopenic).[11,19] Even if feasible, the histopathology examination and culture may be falsely negative because the invasive procedure could not reach the infected area or the patient is already receiving systemic antifungal therapy. Another issue regarding cultural exam is the slowness or absence of growth of fungal isolates.[20] It is clearly urgent the need to search for an alternative diagnostic approach, that may be specific, sensitive and quick. This alone or in complement with radiological tools may improve early diagnosis of IA. Delay is as we know tragic for outcome and most centres develop empiric strategies to overcome the time delay but at toxicity expenses that are also highly expensive. The non-invasive

Table 4. Sensitivity and specificity of GM and PCR assays.

	PCR/(95% CI)	GM/(95% CI)
Sensitivity %	75,0/(50,6–90,4)	87,5/(46,7–99,3)
Specificity %	91,9/(86,5–95,3)	93,1/(87,8–96,4)
PPV %	51,7/(32,9–70,1)	38,9/(18,3–63,9)
NPV %	97,0/(92,7–98,9)	99,3/(95,8–99,8)

Abbreviations: PCR, polymerase chain reaction; GM, galactomannan; PPV, positive predictive value; NPV, negative predictive value.

serum markers *Aspergillus* GM test, PCR detection for fungal DNA and 1,3-β-D-glucan test can overcome some of these problems. For budget reasons the majority of the centres have implemented one test only and sometimes it is hard to choose. Both GM and 1,3-β-D-glucan are available kits, standardized which allow for comparison between centres but very expensive and only screen for *Aspergillus spp*. On the other hand, pan-fungal PCR is broader, it detects any fungal infection, less expensive and very easy and quick to perform.

The aim of this study was to compare two methods for IA screening, serum PCR versus GM in similar cohorts of patients. Our results showed that the GM assay sensitivity was slightly higher than that of PCR (87.5% and 75% respectively), but the difference was not statistically significant which may be explained by the method characteristic, since a pan-fungal PCR screening will detect any other fungal infection that when validated for IA criteria will underestimate IA infection. PCR was highly specific (91, 9%) as the GM assay (93.0%).

An important point to mention is that in our study, 2 consecutive positive serum samples, with both methods, were associated with the development of IA. A single positive sample was not as highly predictive. This strengthens the importance of requiring 2 consecutive serum positive results as a clear indicator of IA[21] by minimizing the risk of false positives.

In this study patients with 2 or more serum GM/PCR positive samples who we were able to perform a bronchoscopy had also positive BAL results reinforcing the notion that BAL GM/PCR determination is a sensitive and specific test for diagnosis of proven and probable IA in whatever fluid or whole blood and may be helpful to confirm diagnosis of IA in these cases.[22–24] The BAL GM/PCR positivity obtained in those patients without serum GM/PCR positivity could be due to a very early diagnosis i.e. before the *Aspergillus* could breach the alveolar capillary surface and enter the bloodstream. The GM cut-off of 0.5 ng/mL used in our laboratory increases the sensitivity at the cost of lowering the specificity[25] but was agreed as the ideal threshold by the EORTC/MSG criteria.

Another observation worthy of mention is that PCR negativation or GM index normalization during

antifungal treatment was associated with a better clinical outcome and survival proving that both methods are useful for therapy monitoring. Both GM and PCR methods are simple, safe, fast and reliable. Both have acceptable sensitivity and specificity and can be used to decide to start pre-emptive antifungal therapy. Both tests enable us to follow up the evolution of the disease by monitoring the efficacy of the therapy chosen and change to alternative antifungal drugs in case of no response but antifungal treatment duration should be determined not solely by normalization of serum GM/PCR but also by the clinical and radiologic findings resolution,[26,27] in fact, survival is significantly better in patients who become GM negative.[28,29] In our study PCR based approach was identical but more prospective studies are needed to validate these results.[30,31] The PCR assays have not yet been standardized and remain investigational, although coordinated efforts through the EORTC/MSG to address the issue of standardization of nucleic acid–based testing are ongoing.[32,33] Quantitative techniques in particular real-time PCR may improve sensitivity.[34,35] The advantage of a commercial PCR diagnostic kit would allow for standardizing methodology between centres but will increase costs.[36,37] PCR results are not interfered by diet or antibiotics administration as with GM tests where false positives can be caused by several items such as the passage of dietary through injured mucosa (pasta, rice, canned vegetables),[38] by the administration of antibiotics like piperacillin-tazobactam and amoxicillin-clavulanic acid,[39–41] by Bifidobacterium lipoteichoic acid[42] and some other fungal species including *Penicillium, Paecilomyces, Histoplasma and Geotrichum*[43–45] and by gastrointestinal chronic graft-versus-host disease.[46] Furthermore, variables that reduce the fungal load and thus the circulating GM levels, such as mould-active antifungal prophylaxis decrease the assay performance.[47–49] BAL GM is a valuable adjunctive diagnostic tool generally not hampered by these variables but the lack of widespread experience and consensus on optical density (OD) cut-offs and standardized method of collection limits at some point its utility.[50] An electrolyte solution (containing sodium gluconate produced by *Aspergillus niger* fermentation) used for bronchoalveolar lavage (BAL) can also cause false positives.[51]

Delayed IA diagnosis is associated with poor prognosis and high mortality rates. Antifungal therapy should be initiated as soon as IA is suspected i.e. at the stage of possible infection, because it improves outcome,[52,53] reduces the use of antibiotics and antifungals, is less toxic and is cost-effective. Non-culture based biomarkers could expand our capabilities to establish early and specific diagnosis for IA or even help with assessment of response to antifungal therapy. GM is for the time being a better option. To improve its diagnostic accuracy the number of samples per episode of neutropenia should be increased. Ideally the same patient should be tested by PCR and GM simultaneously at the same time but we doubt that such a study will ever be performed routinely since it implies more costs and the majority of the departments have to answer to tight budgets.[54,55]

Author's Contributions

RL contributed to the conception and design of the study; acquired, analysed and interpreted the data; performed statistical analysis; reviewed the literature; wrote, organized and revised the manuscript. PR designed the main study; performed PCR analysis; acquired, analysed and interpreted the data; revised the manuscript. TF2 performed the galactomannan assay. NA, TF1, AM, FC, JC, MS, LC, GF, AC, EC, MHS, ASR, IC, JV acquired, analised and interpreted the data. ABS contributed to the study conception and design; acquired, analysed and interpreted the data; revised the manuscript.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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