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PCR as a Screening Test for Invasive Aspergillosis in Haematological Patients: A Pilot Study

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Abstract Invasive aspergillosis is a leading cause of morbidity and mortality in immunocompromised patients, particularly in individuals with haematological malignancy and in haematopoietic stem cell transplant recipients. Nowadays, the galactomannan (GM) assay has been widely used as an indication of invasive aspergillosis, even though the test is known to generate false-positive results. The aim of this study was to compare the performance of GM and real-time PCR (qPCR) to detected *Aspergillus* in blood samples obtained from high-risk haematological patients. Haematological patients were screened twice weekly with GM testing, which was performed by the Platelia ELISA kit. An additional sample of whole blood (4 ml) was obtained for the purpose of qPCR testing. Sixty-four samples from 12 patients with haematopoietic stem cell transplant or haematological malignancy were studied. The overall accordance between GM and qPCR tests was 96.9 % (62 samples). Only two samples showed contradictory results, with positive GM test and negative real-time PCR results. Based on the high concordance between GM and qPCR in terms of negative results, the main utility of qPCR could be in the confirmation of positive results seen with GM testing.

Keywords Invasive aspergillosis · Haematological patients · Screening

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Introduction

Invasive aspergillosis (IA) is the most common invasive fungal disease in immunocompromised patients, with high mortality rates [1]. The microbiological diagnosis of IA was based for decades on the recovery of *Aspergillus* in culture. However, this method is known to have poor sensitivity, frequently resulting in a late diagnosis [2]. Galactomannan (GM) assay has been widely accepted as a biomarker for the presence of *Aspergillus* in clinical samples. Despite providing an early diagnosis of IA, GM testing can be associated with a high frequency of false-positive

results [3]. Molecular assays such as real-time polymerase chain reaction (qPCR) have shown potential as a screening and diagnosis test in medical mycology [4], even though these techniques are hampered by limited standardization [5]. In the last years, several efforts have been conducted in an attempt to standardize the technical steps involved in qPCR, particularly related to the complexity of *Aspergillus* DNA extraction [6]. In order to determine the performance of GM and qPCR, here we prospectively screened high-risk haematological patients by using a combination of the two tests, in a single medical centre in southern Brazil.

Patients and Methods

This was a cohort study performed in a reference centre for haematological malignancies and stem cell transplantation, Hospital de Clínicas de Porto Alegre (HCPA). As a part of their routine medical care, patients with haematological malignancies or stem cell transplant recipients were screened twice weekly with GM testing. An additional sample of whole blood (4 ml in EDTA tubes) was obtained for the purpose of qPCR testing. All but one patients (acute lymphocytic leukaemia) were cared for in HEPA filter units. Patients were followed by a median of 27.5 days after entering the study (range 1–119 days). Informed written consent was obtained from all patients, and the study was approved by the local Ethic Committee.

The presence of GM was determined by using the Platelia Aspergillus GM ELISA kit (Bio-Rad), according to manufacturer's instructions. The cut-off optical density to define positivity was 0.5.

DNA was extracted from whole blood as previously described [6]. Briefly, the protocol involved hypotonic lysis of red blood cells, followed by chemical lysis of white blood cells and mechanic rupture of cells with bead beating. A volume of 200 µl of the supernatant was transferred to Maxwell® automated extraction. The final elution volume was 70 µl.

Real-time PCR reaction was performed using the qPCR Alert Amplimaster Kit (Nanogen®, Italy) in a 7500 Real-Time PCR system equipment (Applied Biosystems®). This is a quantitative qPCR commercial test in which an internal control is also included (human beta-globin). All reactions and cycling conditions were performed according to the manufacturer's instructions. PCR positivity was considered when amount of DNA was greater than seven genomic copies, according to the manufacturer's recommendation. Physicians were all blinded to the qPCR results, and the laboratory staff had no access to the GM results.

Results

A total of 64 samples from 12 patients were studied. These individuals were allogeneic hematopoietic stem cell transplant recipients ($n = 9$) or had haematological malignancies ($n = 3$). They were predominantly male (58.3 %), with a median age of 49.5 years (range 23–64 years). Neutropenia was presented in 75.0 % ($n = 9$), including 2 patients with <100 neutrophils/mm^3. The overall concordance between GM and qPCR tests was 96.9 % (62 samples). GM was negative in 61 samples (95.3 %) and qPCR in 63 samples (98.4 %). Both tests were positive in only one sample, with a GM optical index of 0.51, whilst PCR indicated a burden of 72.3 genomic equivalents/ml. This patient had probable IA, with *Aspergillus fumigatus* recovered in culture of a nasal biopsy (Table 1).

In two samples obtained from a single patient, GM and qPCR tests showed contradictory results, with positive GM (indices of 0.67 and 2.95) and negative qPCR results. This patient had no tomography findings suggesting IA, and he was lately found to be infected by *Paecilomyces variotii*. He had also received several courses of antimicrobial treatments, including vancomycin, meropenem, amoxicillin and penicillin. One additional patient had a positive culture obtained from laryngeal mucosa that was positive for *Rhizopus* spp.—both GM and qPCR were negative in this case.

Discussion

In this prospective pilot study, a strong concordance was observed between GM and qPCR tests, when screening haematological patients for IA. In particularly, an excellent agreement (95.3 %) was observed regarding negative results, suggesting the absence of benefit of adding a second test such as qPCR when serum GM is negative.

In the past, fungal identification was based on morphological features only. However, detection solely based on culture and microscopy is difficult,

Table 1 Demographic and clinical characterization of patients with allogeneic hematopoietic stem cells or haematological malignancy included in this study

ID	Sex	Age	Neutropenia	Positive microbiology findings	GM	qPCR	Interpretation
1	M	24	No	–	Negative	Negative	No fungal infection
2	F	49	<500	Positive culture for *Paecilomyces variotti*	Positive	Negative	False-positive GM result
3	F	23	No	–	Negative	Negative	No fungal infection
4	M	62	<500	–	Negative	Negative	No fungal infection
5	M	51	No	Positive culture for *Rhizopus*	Negative	Negative	Real-negative GM result
6	M	59	<500	–	Negative	Negative	No fungal infection
7	F	56	<500	Positive culture for *Aspergillus fumigatus*	Positive	Positive	Invasive aspergillosis
8	F	50	<500	–	Negative	Negative	No fungal infection
9	M	48	<500	–	Negative	Negative	No fungal infection
10	F	41	<100	–	Negative	Negative	No fungal infection
11	M	48	<500	–	Negative	Negative	No fungal infection
12	M	64	<100	–	Negative	Negative	No fungal infection

Legend: *ID* patient identification, *F* female, *GM* galactomannan, *M* male, *qPCR* quantitative real-time PCR test

and trained staff are required to attain accurate identification [3]. Molecular methods have shown to be more reliable to distinguish fungal species [7], even though no nucleic acid-based test has been accepted as a standard diagnostic tool for IA [8]. Several attempts to detect *Aspergillus* DNA in clinical samples have been made in the last years, but most techniques are challenged by the difficulty in obtaining good-quality fungal DNA. It is well known that extraction efficacy is influenced by sample type, the amount of clinical material and the protocol used [8, 9]. In this study, we performed a DNA fungal extraction with mechanic disruption using ceramic beads, followed by automated DNA extraction, in an effort to improve the recovery of *Aspergillus* DNA in whole blood. As previously published [5, 6], this is currently the most promising method to extract *Aspergillus* DNA from whole blood, potentially allowing qPCR to be used a screening test for patients at risk for IA.

The use of a combination of non-culture-based diagnostic tests in patients at risk for IA has already been suggested by others [7, 10, 11]. Rogers et al. [12] evaluated the utility of combining GM and qPCR tests and recommended the use of a second confirmatory assay different from GM for the detection of IA. This confirmatory test could be qPCR, which could facilitate the interpretation of a false-positive GM assay [12, 13]. Our study was limited by the small number of samples studied. However, data clearly show an elevated degree of concordance regarding negative results.

In conclusion, this study pilot suggests that qPCR testing may be an interesting diagnostic tool when screening haematological patients for IA. In particular, qPCR may be useful to confirm positive results with GM testing. Using a standardized protocol for DNA extraction is a critical step to apply qPCR as a screening test in the clinical laboratory. Confirmation of these findings in large prospective studies is ultimately required.

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Conflict of interest In the past 5 years, Dr Pasqualotto has received research grants and given paid talks for Pfizer, MSD, Astellas, United Medical (Gilead) and Bagó. All other authors have no conflict of interest to declare.

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