

Diagnosis of invasive aspergillosis: Application of Polymerase Chain Reaction Techniques (PCR) and Enzyme-Linked Immunosorbent Assay for Detection of Galactomannan (EIA-GM[®])

Diagnóstico da aspergilose invasiva: Aplicação das Técnicas de Reação em Cadeia da Polimerase (PCR) e Ensaio Imunoenzimático de Detecção da Galactomanana (EIA-GM[®])

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ABSTRACT

Introduction: invasive aspergillosis (AI) is a serious fungal infection caused by species of the genus *Aspergillus* that primarily affects leukemic and diabetic patients and those recipients of stem cell transplants, which have neutropenia. The fungi spores that colonize the lung epithelium may invade the endothelial cell lining and vascular access and thus, spread to other organs through the blood. The high mortality of the disease is related to severe immunosuppression, rapid infection progression, and especially lack of an early and efficient diagnosis. Therefore, the diagnosis in the initial infection phase is beneficial, providing a more effective therapy that can reduce the disease's mortality rate. **Objective:** this study aimed at evaluating the applicability of the polymerase chain reaction (PCR) in assisting the diagnosis of AI compared to the results generated by galactomannan enzyme immunoassay (EIA-GM[®]) that is already commercially validated. **Methods:** 245 samples from patients treated in the Santa Casa de Belo Horizonte hospital were analyzed. Among these samples, 16% (N = 39) were positive in EIA-GM[®] tests. Subsequently, these 39 positive samples were analyzed by PCR. **Results:** According to the results, the PCR technique showed 97.44% sensitivity, 97.96% accuracy, and 100% specificity compared to EIA-GM[®]. **Conclusion:** the PCR technique may aid in the diagnosis of AI, always associating the results to the patient's clinical and immunoassay tests. **Key words:** Invasive Pulmonary Aspergillosis; *Aspergillus*; Immunoenzyme Techniques; Polymerase Chain Reaction.

RESUMO

Introdução: a aspergilose invasiva (IA) é uma infecção fúngica grave causada por espécies do gênero *Aspergillus* e acomete principalmente pacientes leucêmicos, diabéticos e aqueles receptores de transplante de células-tronco, que apresentem neutropenia. Os esporos dos fungos que colonizam o epitélio pulmonar podem invadir as células endoteliais de revestimento e o acesso vascular e, assim, disseminar-se para outros órgãos através do sangue. A elevada mortalidade da doença está relacionada à imunossupressão grave, à rápida progressão da infecção e, principalmente, à ausência de um diagnóstico precoce e eficiente. Portanto, o diagnóstico na fase inicial da infecção é adequado, proporcionando uma terapia mais eficaz, o que pode reduzir a taxa de mortalidade da doença. **Objetivo:** o presente estudo teve em vista avaliar a aplicabilidade da técnica de reação em cadeia da polimerase (PCR) no auxílio do diagnóstico de AI, em comparação com os resultados gerados pelo ensaio imunoenzimático de galactomanana (EIA-GM[®]), este já validado comercialmente. **Métodos:** foram analisadas 245 amostras de pacientes tratados no hospital Santa Casa de Belo Horizonte. Entre essas amostras, 16% (N = 39) foram positivos nos testes EIA-

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-GM®. Em seguida, essas 39 amostras positivas foram analisadas pela técnica de PCR. **Resultados:** de acordo com os resultados, a técnica de PCR apresentou taxa de 97,44% de sensibilidade, 97,96% de acurácia e 100% de especificidade, quando comparada ao método EIA-GM®. **Conclusão:** a técnica de PCR pode auxiliar no diagnóstico da AI, sempre associando os seus resultados à clínica do paciente e aos testes de imunoenaios.

Palavras-chave: *Aspergilose Pulmonar Invasiva/ diagnóstico; Aspergillus; Técnicas Imunoenzimáticas; Reação em Cadeia da Polimerase.*

INTRODUCTION

Invasive aspergillosis (AI) is caused by species of filamentous fungi of the genus *Aspergillus*. Infection occurs by inhalation of conidia that lodge in the lungs, may invade the endothelial cell lining, and spread to other organs through the blood stream.¹

According to some published studies, the mortality rate of the disease ranged from 40 to 90%.¹⁻⁶ These variations may occur due to the applied treatment regimen and a few interaction factors between host and fungus such as patient's immune status, virulence of the infecting strain, and processes that contribute to the disease pathogenesis.^{1,6} The advanced chronic obstructive pulmonary disease (DPOC) as well as the prolonged use of corticosteroids is also associated with a high risk of AI.^{7,8}

Pediatric patients with cancer, particularly those with hematologic malignancies, present a high risk of developing AI. In these patients, the mortality rate is 80-90%.²

The AI high mortality rate is also related to difficulties in obtaining an early disease diagnosis.^{9,10} The diagnosis has rarely been established during the initial phase of infection when the patient has low fungal load and when therapy would be efficient.⁵ Several studies have warned that survival can be enhanced by an early diagnosis, followed by immediate treatment.^{3,10,11}

Considering the lack of certainty in diagnosis and the high rate of mortality associated with the disease, the use of empirical antifungal therapy has been advocated in particular for neutropenic patients who manifest fever and are unresponsive to broad-spectrum antimicrobial treatment.^{3,9,10,12} However, this recommendation is not yet securely validated and almost inevitably results in overtreatment, toxicity, additional costs, and changes in the microbial ecology.³

The development of new methods to assist in the diagnosis of AI is needed because traditional myco-

logical methods, such as culture and microscopic identification of fungal species, are usually time-consuming.^{2,5} Moreover, obtaining sample material using invasive procedures may be difficult, particularly in pediatric patients and, also, the culture of blood is not useful for the detection of *Aspergillus spp.*^{2,5} Thus, the diagnosis can be improved by the detection of galactomannan (GM) in the blood stream of patients.^{2,5,13}

The galactomannan enzyme immunoassay detection assay (EIA-GM®), a polysaccharide in the cell wall of *Aspergillus* that may be present in the serum and other biological fluids of patients with AI, has excellent negative predictive value and can exclude this disease with high probability.^{9,10} Its high sensitivity enables the disease diagnosis to be defined even before it is clinically manifested.^{5,6}

The EIA-GM® (Platelia®*Aspergillus*, Bio-Rad Laboratories, France) is an already marketed test that has been used extensively worldwide as a predicting AI diagnosis in immunocompromised patients.^{12,14,15} A study demonstrated sensitivity of 65.7% and 95% confidence interval and concluded that circulating GM is predictive of AI in most pediatric oncology patients, and the GM antigenemia test may precede the AI clinical, microbiological, or radiographic evidence.¹³

In Europe, the test was introduced in the 1990s and the Food and Drug Administration (FDA) approved it in 2003 in the USA.¹² The test is performed in sequential steps and can be completed in about four hours providing a numerical result that can be grouped into negative or positive. The serial sampling must be performed at least twice a week while risks persist.^{2,14-19}

Molecular biology techniques also have great potential in aiding AI diagnosis. The polymerase chain reaction (PCR) is an alternative diagnostic and minimally invasive method that has been increasingly used in the diagnosis of systemic mycosis.²⁰ This method is an important tool because it can detect circulating DNA from the causative agent at very low levels, reaching picograms/ml of DNA in a clinical sample.^{20,21} Studies that demonstrate the feasibility of PCR for the diagnosis of AI by detecting *Aspergillus* DNA in blood have demonstrated a sensitivity of 36 to 98% and specificity of 72 to 100%.^{22,23} In another research, PCR proved to be less sensitive than the EIA-GM®, 45 detections versus 93%, respectively,^{18,24-26} suggesting the combination of both tests (EIA-GM® and PCR) to achieve rapid diagnosis in neutropenic immunocompromised patients.²

This study aimed at assessing the implementation of PCR as an aid in the diagnosis of AI, comparing results with the EIA-GM[®] enzyme immunoassay, which is already a commercially validated and widely used test.

METHODOLOGY

Biological samples

A total of 245 samples of peripheral blood were collected from patients with suspected AI and treated at Santa Casa of Misericórdia Hospital of Belo Horizonte (HSCMBH) between March of 2012 and March of 2014.

All samples were analyzed by the EIA-GM[®] enzyme immunoassay for the detection of galactomannan; positive samples were subjected to polymerase chain reaction (PCR).

The project was approved by the Research Ethics Committee (CEP) of HSCMBH (protocol: 099/2011).

Platelia *Aspergillus* immunoassay (EIA-GM[®])

The GM[®] EIA-kit was used for detecting circulating GM in the patient's blood; the method was performed according to the manufacturers' instructions.

DNA Extraction from whole blood

Positive samples for the *Platelia Aspergillus* immunoassay test were analyzed by polymerase chain reaction (PCR) in order to compare results between the two techniques. For this, DNA was extracted from whole blood using the Invitrogen (USA) kit and according to the manufactures' instructions.

Once DNA was extracted and purified, it was measured by spectrophotometry using the NanoVue Plus instrument (GE Healthcare Life Sciences, Sweden) and, regardless of the concentration of purified DNA, all samples were diluted ten times with sterile ultrapure water before being used in PCR reactions.

In order to detect possible cross-reactions with other regions of human DNA, a negative control group composed of five blood samples from patients negative for AI was included in the study.

Nested-PCR

DNA extracted from whole blood was subjected to nested PCR, which consists of PCR performed in two steps, thus increasing the amount of amplified product and resulting in improved gel electrophoresis visualization. The following primers for the conserved region of the 18S rDNA in dimorphic fungi were used as: sense – V9G (5'-TTACgTCCCTgCCCTTTgTA-3') and antisense – LS266 (5'gCATTCCCAAACAACCTgACTC-3').²⁷ Positive and negative controls were included in all PCR reactions. The positive controls were DNA samples from cultured *Aspergillus niger* and *Aspergillus fumigatus* from the collection of the Mycology Laboratory cultures at the Santa Casa of Belo Horizonte. Tubes containing only sterilized ultrapure water and no DNA samples were used as negative controls.

The final amplification product of the first step PCR (PCR-out), which generated a fragment of 800 base pairs (Bp), was analyzed in 7% polyacrylamide gel and stained with 2% silver nitrate. Specific primers for the constitutive β -globin gene were used in all reactions as quality control. The following reagents were used in each reaction: 2 mM MgCl₂; 200 mM dNTPs; 0.6 mM of each primer (Sigma, USA); 1 IU Platinum Taq DNA Polymerase, specific PCR buffer (Invitrogen, USA); and 20 ng of DNA. The cycle used was: one cycle of five minutes at 94 °C; 40 cycles of 30 seconds at 60 °C; 30 seconds at 72 °C; and 30 seconds at 94 °C, and one cycle of 7 minutes at 72 °C.

The second step of PCR (PCR-in) used the product amplified in the first step. All amplified products from samples and controls were diluted 1:10 and the volume of 1 to 2 μ L were added to the PCR reactions as described above. The primers used were: sense – ITS1 (5'-TCCgTAggTgAACCTgCgg-3') and antisense – ITS4 (5'-TCCTCCgCTTATgATATgC-3').²⁷

These primers amplify the region already amplified before in the PCR-in step allowing an improved visualization of bands. The final product, a fragment of approximately 600 Bp, was observed in 7% polyacrylamide gel stained with 2% silver nitrate (Table 1). The "100 Bp" molecular weight marker was used in each gel (Ludwig brand, Brazil) where 5 μ L of samples mixed with 5 μ L of dye buffer were applied (Promega, USA).

Table 1 - Main established epidemiological data

Established epidemiological data	
Analyzed samples (units)	26
Male (%)	49.00%
Female (%)	51.00%
Calculated mortality rate (%)	30.00%
Average age (years)	38
Hospitalization average time (months)	2
Prevalent skin color (HSCMBH classification)	Parda
Prevalent underlying disease (CID-10)	C920
Average antimicrobials used during hospitalization	8
Use of injectable and/or tablet voriconazole (%)	35.90%
Use of Amoxicillin/Clavulanic acid and/or Piperacillin/Tazobactam – injectable (%)	15.40%

CID: International Classification of Diseases; CID C920: acute myeloid leukemia.

RESULTS

A total of 245 samples from patients attending services at the Santa Casa of Misericórdia of Belo Horizonte between March of 2012 and March of 2014 were analyzed, among these, 16% (N = 39) were positive in the (EIA-GM®) enzyme immunoassay.

The epidemiological data and use of antimicrobial drugs by these positive patients contained in the computerized hospital system were provided by the Hospital Infection Control Commission (CCIH); 33.3% (N = 13) were not found. Thus, the epidemiological analysis compiled in Table 1 was performed in only 26 patients.

A diagnostic or screening test was carried out to compare the results generated by PCR and the (EIA-GM®) enzyme immunoassay using the Epidemiologic Statistics for Public Health® Open Source program available at <http://www.openepi.com> in order to establish sensitivity, specificity, and accuracy (precision) of the nested PCR technique compared to the EIA-GM® detection test.

The sensitivity of the nested PCR test was 97.44%, with an accuracy of 97.96, and 100% specificity (Table 2).

DISCUSSION

According to the analysis of medical records, most patients were females (51.0%), with a mean age of 38 years. Regarding skin color, most were brown. The predominant underlying disease was acute myeloid leukemia.

Table 2 - Analysis of nested PCR test performance compared to the EIA-GM® enzyme immunoassay using the Epidemiologic Statistics for Public Health® Open Source program (<http://www.openepi.com>)

Open Source Epidemiologic Statistics for Public Health	Platellia GM detection test			
	Positive	Negative		
Nested-PCR	Positive	38	0	
	Negative	1	5	
Total		39	5	
Specificity	Sensitivity	Accuracy	VPP	VPN
100%	97.44%	97.73%	100%	83.33%

The leukemic patient already presents immunosuppression and, when hospitalized, is exposed to pathogens either in the hospital environment or through individual therapeutic practices that are indispensable for their recovery, for example, the use of intravenous access for the administration of drugs. Thus, the patient is at high risk for contracting AI and about 80% of leukemia patients manifest infectious complications at some point during the course of their disease.²⁸ Therefore, it is important to take preventive measures with an early detection of AI in these patients.

The average hospital stay was two months. The treatment used by patients hospitalized with leukemia was distributed as nine (35%) using injectable and/or injectable compressed voriconazole as the treatment base of this type of cancer. Only four patients (15.4%) used amoxicillin/clavulanate and/or piperacillin/tazobactam. Other 13 (50%) patients underwent another therapeutic treatment.

According to the results of this study, the *Nested* PCR presented 97.44% sensitivity, 97.96% accuracy, and 100% specificity when compared to the EIA-GM® method.

In a study by Martín-Rabadán *et al.*⁴, the EIA-GM® galactomannan test presented a sensitivity of 78% (61-89%) and specificity of 81% (72-88%) using a GM optical density index (GMI) of 0.5 ng/mL as the cutoff.⁴ Thus, it is demonstrated that the screening of *Aspergillus spp* genome in the blood stream of patients by the nested PCR test may be more sensitive than the EIA-GM® galactomannan test, aiding in the early diagnosis of AI in patients with suspected AI. In this study, the primers used in the PCR reactions were specific for the genus *Aspergillus*, which makes it a highly specific technique. However, the diagnostic value only based on the results generated by PCR is still a topic for debate.

According to Martín-Rabadán *et al.*⁴, the situations of false-positive for the galactomannan testing through EIA-GM® can be classified into four groups:

- cross-reactivity with infection by other fungi;
- intravenous administration of products derived from fungi such as beta-lactam antibiotics or presence of gluconate electrolyte solution for hydration;
- lack of laboratory quality control of samples in the post-extraction;
- enteral absorption of a substance containing GM or bacterial antigens (these have been suggested, but not proven).

Furthermore, different studies demonstrate that false-positive results can also occur in patients affected by other systemic mycoses because the epitope detected by the monoclonal antibody used in the Platelia GM® EIA-test is also present in other similar antigens and some fungal species, being them:

- **genera:** *Paecilomyces*, *Alternaria*, *Trichophyton*, *Botrytis*, *Walleimia*, *Cladosporium*, *Geotrichum*, *Trichothecium*, *Myceliophthora*, *Blastomyces*, and *Fusarium* infections caused by *Neosartoria*;^{4,16,17,23,29-31}
- **species:** *Paracoccidioides brasiliensis*, *Histoplasma capsulatum*, *Cryptococcus neoformans* e *Cryptococcus gattii*, *Geotrichum capitatum*, *Penicillium marnettei*.^{15,29}

The high mortality rate of the disease in high-risk populations reflects, in part, the difficulty in establishing a reliable early diagnosis and the rapid and accurate identification necessary for the successful clinical management of infection.^{3,11} In the present study, the mortality rate of 30% was observed among patients positive for EIA-GM, AI patients, treated at the Santa Casa of Belo Horizonte Hospital. Due to the low mortality rate among AI patients in this hospital compared to other published studies, the importance of the structure in public and private hospital services for the sequential measurement of serum levels of galactomannan and the clinical benefits for patients with an early AI diagnosis can be inferred. The data analysis reinforces the findings by others, suggesting that the prospective screening for GM may precede clinical, microbiological, or radiographic evidence of AI, and thus, allow an early diagnosis between eight and nine days.^{3,13,14}

The ability to quickly detect the GM antigen in sera of patients or to detect the fungus genome in total peripheral blood is of great importance for an early and rapid diagnosis of AI and, hence, to an effective therapeutic intervention. However, the results generated by these markers need to be associated with laboratory and clinical parameters.

The *Nested* PCR results are significant and indicate a path for its routine use. However, more studies are necessary using a more representative sample size of the population's prevalence/incidence to validate this method as an effective diagnosis tool, as well as testing its reproducibility. Despite the relatively small sample, this study presented relevant and consistent results with other published studies, showing that this technique may contribute to the decision-making process of clinical staff to the current situation of AI.^{30,32}

Novaretti et al.³¹ also showed that the PCR-*Nested* test has considerable clinical potential for the early diagnosis of AI. This research aimed to standardize the *Nested* PCR for the detection of *Aspergillus spp* in 23 leukemia patients. Out of the studied individuals, six (21.7%) presented positive results in the nested PCR for *Aspergillus*, five of them with pulmonary involvement. The clinical evidence of infection by *Aspergillus* could not be observed in one of the samples that tested positive by the PCR method. The sensitivity and specificity of this test were 100 and 94.4%, respectively.

Nested PCR is confirmed to be a highly sensitive diagnostic tool for the detection of microorganisms in various biological samples, including fungi because the use of two steps PCR increases the method's sensitivity. However, it has the limitation of facilitating contamination with ubiquitous conidia or co-amplification with human DNA because it also increases the probability of contamination with other genomes. Therefore, testing negative controls (human DNA and ultrapure water) in each PCR reaction is essential, as performed in this study.³³

This study contributes to the clarification of the status of invasive aspergillosis, which is worrying doctors and scientists, including those in developed countries that regardless of the advances in Medicine, face the problem of systemic mycoses. The application of this research will provide advances in the diagnosis and treatment of systemic mycoses in both private and public health services.

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The authors have no conflict of interest to disclose.

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