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ORIGINAL ARTICLE

EPIDEMIOLOGICAL EVALUATION OF PATIENTS SUBMITTED TO THE GALACTOMANNAN TEST WITH SUSPECTED INVASIVE ASPERGILLOSIS

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ABSTRACT

Invasive aspergillosis (IA) increases dramatically when there is potential risk in many patient groups, in particular with hematological malignancies. The purpose of the study was to trace the epidemiological profile of patients who underwent galactomannan test by ELISA with suspected IA and to determine the factors that contribute to the development of the disease. We evaluated 264 patients who underwent galactomannan test with suspected IA from 2013 to 2015. The clinical-epidemiological characteristics were determined using descriptive statistics. The variables were evaluated using the chi-square test (χ^2) and the G-test, with p-value considered significant below 0.05. According to the classification for IA by the European Organization for Research and Treatment of Cancer, the disease was considered proven in 7.3%, defined by positive culture for the fungus, 6.4% as probable through detection of galactomannan and the presence of pulmonary infiltrates and 5.1% as possible by radiological alterations suggestive of IA and negative galactomannan test. The mortality rate was 31.6% of all patients and 61.3% for proven / probable / possible IA indicating that the disease was significantly associated with the risk of death. According to these result indications and considering the high mortality rate caused by the development of IA, as well as the fact that early therapy promotes significant improvement in the patients' prognosis, we conclude that the detection of galactomannan may be considered an effective method to aid the identification of IA.

KEY WORDS: Invasive pulmonary aspergillosis; neutropenia; enzyme-liked immunosorbent assay; ELISA test searching for *Aspergillus*; galactomannan.

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INTRODUCTION

Invasive aspergillosis (IA) is an opportunistic fungal infection mainly affecting immunocompromised patients and, despite continuous therapeutic improvements with the use of new antifungal drugs, represents an important cause of morbidity and mortality, which in predisposing patients, may currently exceed 50%. This rate is decreasing annually due to better diagnostic conditions, therefore allowing the early start of treatment with antifungals. (Brown et al., 2012; Klingspor et al., 2015; Neofytos et al., 2013; Ramírez & Garnacho-Montero 2018; Solano & Vázquez, 2018).

Several species of *Aspergillus* may evolve into an invasive disease in profound neutropenic and/or critically ill patients due to the concurrence of multiple predisposing factors. Thus, it includes a potential IA risk for patients with hematological malignancies with prolonged neutropenia, patients with transplanted allogeneic hematopoietic stem cell, inherited or acquired immunodeficiency, and patients undergoing corticosteroid therapy (Patterson et al., 2016; Rotjanapan et al., 2018). IA has recently been associated with viral pulmonary infections, such as influenza and COVID-19 (Alanio et al., 2020; Waldeck et al., 2020). In this sense, the exposure to hyphae or spores of the *Aspergillus* species, such as *Aspergillus fumigatus*, presents the most important factor to immunocompromised patients regarding the development of high risk IA (Klingspor et al., 2015; Pagano et al., 2011).

IA patients usually present non-specific clinical signs and radiological findings in the early phase of disease, however infection becomes apparent at advanced stages (Colombo et al., 2017). In addition, IA can disseminate and potentially affect any organ, where lungs represent the primary infection site as conidiae of *Aspergillus* are easily aerosolized, being easily inhaled (Panackal et al., 2010).

Proper diagnosis of IA requires direct visualization of the acute branching septate hyphae through microscopic examination or tissue biopsy and recovery of the fungi in specific culture media (De Pauw et al., 2008). However, for the critically ill, such as neutropenic and onco-hematological thrombocytopenic patients, these diagnostic alternatives for invasive fungal infection by *Aspergillus* species are not possible, since they are dangerous, time-consuming and the culture yield is variable, signifying that most cases of invasive aspergillosis in these patient groups are deemed probable or possible (De Pauw et al., 2008; Lamoth & Calandra, 2017). Therefore, the galactomannan (GM) enzyme immunoassay that detects polysaccharides in the cell wall of *Aspergillus* species is an important diagnostic noninvasive test, when monitored serially in the serum and/or bronchoalveolar lavage fluid during invasive infection (Fernandes et al., 2017; Lamoth & Calandra, 2017).

Although the GM dosage is very useful for the early diagnosis and monitoring of antifungal therapy in the treatment of IA, the importance of combining clinical and microbiological data cannot be disregarded since these play a fundamental role in the detection and evolution of IA. The purpose of this study was, therefore, to trace the epidemiological profile of patients who underwent the galactomannan test with suspected IA treated at a reference hospital in Goiânia, Goiás, Brazil, and to determine the main factors that contribute to the development of this disease.

METHODS

This descriptive epidemiological type study looked at the population of patients who underwent galactomannan serum testing at the Bone Marrow Transplantation Laboratory in the Hematology Department of the Araújo Jorge Hospital (HAJ), belonging to the "Associação de Combate ao Cancer em Goiás" (ACCG) in Goiânia, Goiás, Brazil. The study was approved by the Research Ethics Committees of the Pontifical Catholic University of Goiás (Protocol 45376215.0.0000.0037/2015) and the "Associação de Combate ao Cancer em Goiás" (Protocol 45376215.0.3001.0031/2015).

In this study, 264 patients with suspected IA were evaluated, including patients that underwent galactomannan ELISA test (Platelia™ *Aspergillus* Ag ELISA Kit, Bio-Rad Laboratories, Marnes-la-Coquette, France), with the equipment: Multiskan™ FC Microplate Photometer - Thermo Fisher Scientific, Waltham, MA, USA) from 2013 to 2015 with clinical data in medical records at the hospital. Subjects treated in the hematology sector before 2013 or after 2015 were excluded as well as those without follow-up by the hospital.

To prepare the database, a form was filled in with information such as: general and clinical characteristics of each patient, including variables such as neutropenia, use of antifungal and / or antibiotics, diagnosis of IA classified according to the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG), evolution to death, Bone Marrow Transplantation (BMT), type of transplantation, quantitative and qualitative results, among others. The form was devised with the purpose of collecting information necessary to evaluate possible interferences in the ELISA test for galactomannan detection and factors that might be associated with the development of IA in the evaluated population.

The clinical-epidemiological characteristics were determined through descriptive statistics. The relationship between the development of IA and BMT and type of transplantation, or neutropenia found in patients and other parameters such as age, sex, type of hematological disease, and use of antifungal drugs and antibiotics were evaluated using the chi-square (χ^2) and G tests, with a significance level of 95%, therefore with p-value considered significant below 0.05.

RESULTS

The epidemiological and clinical characteristics of the patients are presented in Table 1. The average age of the patients analyzed was 43.7 ($\pm$19.3) years where the vast majority was aged between 30 and 59. One hundred thirty (55.5%) were males, of which 74 (31.6%) died. Regarding BMT, 133 (56.8%) had undergone transplantation, 91 (38.9%) of the autologous type and 42 (17.9%), allogeneic.

Of the patients evaluated, 225 (96.2%) had malignant hematological diseases and 110 (47.0%) were neutropenic, 14 (6.0%) with mild neutropenia, 16 (6.8%), with moderate neutropenia and 80 (34.2%) with severe neutropenia.

According to data collected, 175 (74.8%) used antifungals, 197 (84.2%) used some form of antibiotic and / or antiviral, 108 (46.2%) used immunosuppressive drugs and 7 (3.0%) had used corticosteroids for an extended period. Another variable evaluated was the presence of HIV, where only 2 (0.9%) of the 234 patients evaluated presented the virus, one male and one female.

The quantitative indices of galactomannan present in the patients' serum presented an average of 308.1 ng/mL. The qualitative data show 45 (19.2%) patients with positive results, i.e., greater than 0.5 ng/mL. The EORTC/MSG diagnoses classified as possible, probable, and proven had the following percentages: 12 possible cases (5.1%), 15 probable cases (6.4%), and 17 proven cases (7.3%).

The variables studied associated with the GM index (Table 2) indicated that 28 (68.8%; $p = 0.404$) of the positive results for the test were male patients. A statistically relevant result was detected between patient age and positive testing, in which the positive GM result was associated with patients > 50 years old ($p = 0.043$).

Another variable analyzed, which is associated with the GM test result, is the bilirubin index. Patients who show a serum bilirubin quantification > 1.0 mg/dL are considered positive for this biochemical parameter, while patients with < 1.0 mg/dL are negative. According to the statistical data found, $p = 0.062$ was observed for the association described signifying a biased result was reported for patients with positive GM who presented bilirubin levels above normal.

Table 1. Epidemiological and clinical characteristics of patients with suspected Invasive Aspergillosis, at the Araújo Jorge Hospital in Goiânia, Goiás, Brazil, from 2013 to 2015.

Variables	Female (n=104)		Male (n=130)		Total (n=234)		p-value
	n	f (%)	n	f (%)	n	f (%)	
Age (years)							
Average (SD)	42.9 (18.8)		44.2 (19.7)		43.7 (19.3)		
Minimum – Maximum	5	90	2	85	2	90	
Age (years)							
< 30	31	29.8	31	23.8	62	26.5	
30 a 59	50	48.1	63	48.5	113	48.3	
≥ 60	23	22.1	36	27.7	59	25.2	0.475
Serial Sample							
Average (SD)	3.4 (3.6)		3.8 (3.7)		3.6 (3.6)		
Minimum – Maximum	1	24	1	20	1	24	
Quantitative Result							
Average (SD)	264.7 (--)		342.8 (--)		308.1 (--)		
Minimum – Maximum	0.170	7813.0	0.153	10965.0	0.153	10965.0	
Qualitative Result							
Negative	87	83.7	102	78.5	189	80.8	
Positive	17	16.3	28	21.5	45	19.2	0.404
Neutropenia							
No	51	49.0	73	56.2	124	53.0	
Yes	53	51.0	57	43.8	110	47.0	0.341
Light	8	7.7	6	4.6	14	6.0	
Moderate	8	7.7	8	6.2	16	6.8	
Severe	37	35.6	43	33.1	80	34.2	0.744
Antifungal							
No	29	27.9	30	23.1	59	25.2	
Yes	75	72.1	100	76.9	175	74.8	0.490
Antibiotic and Antiviral							
No	22	21.2	15	11.5	37	15.8	
Yes	82	78.8	115	88.5	197	84.2	0.068

Malignant Hematologic Disease							
No	5	4.8	4	3.1	9	3.8	
Yes	99	95.2	126	96.9	225	96.2	0.732
Transplant							
No	42	40.4	59	45.4	101	43.2	
Yes	62	59.6	71	54.6	133	56.8	0.526
Autologous	43	41.3	48	36.9	91	38.9	
Alogenic	19	18.3	23	17.7	42	17.9	0.976
Immunosuppressive Drugs							
No	52	50.0	74	56.9	126	53.8	
Yes	52	50.0	56	43.1	108	46.2	0.356
Prolonged Corticosteroid							
No	101	97.1	126	96.9	227	97.0	
Yes	3	2.9	4	3.1	7	3.0	0.764
HIV Positive							
No	103	99.0	129	99.2	232	99.1	
Yes	1	1.0	1	0.8	2	0.9	0.578
Death							
No	71	68.3	89	68.5	160	68.4	
Yes	33	31.7	41	31.5	74	31.6	0.912
IA Diagnosis							
Negative	88	84.6	102	78.5	190	81.2	
Possible	5	4.8	7	5.4	12	5.1	
Probable	3	2.9	12	9.2	15	6.4	
Proven	8	7.7	9	6.9	17	7.3	0.263

SD = Standard deviation

Assessment with the serum galactomannan index found that 18 (40.0%; p = 0.420) of the 45 patients with a positive result for GM had severe neutropenia. In regard to the drugs used, 36 (80.0%, p = 0.474) used antifungal and 39 (86.7%; p = 0.788) antibiotics and / or antiviral medication and had results higher than 0.5 ng / mL for the GM test.

Table 2. Variables associated with serum galactomannan index, of patients with suspected Invasive Aspergillosis, at the Araújo Jorge Hospital in Goiânia, Goiás, Brazil, from 2013 to 2015.

Variables	Galactomannan Serum Index				p-value
	Negative < 0.50 (n=189)		Positive ≥ 0.50 (n=45)		
	n	f (%)	n	f (%)	
Gender					
Female	87	46.0	17	37.8	
Male	102	54.0	28	62.2	0.404
Age (years)					
≤ 20	20	10.6	11	24.4	
21 a 50	92	48.7	20	44.4	
> 50	77	40.7	14	31.1	0.043
BMT					
Alogenic	31	16.4	10	22.2	
Autologous	78	41.3	13	28.9	0.243
No	80	42.3	22	48.9	
Bilirubin					
≤ 1.0 mg/dL	93	49.2	16	35.6	
> 1.0 mg/dL	41	21.7	16	35.6	0.062
NR	55	29.1	13	28.9	
Neuropenia					
Light (>1000)	12	6.3	1	2.2	
Moderate (500-1000)	13	6.9	3	6.7	
Severe (<500)	62	32.8	18	40.0	0.420
No neutropenia	102	54.0	23	51.1	
Antifungal					
No	50	26.5	9	20.0	
Yes	139	73.5	36	80.0	0.474
Antibiotic + Antiviral					
No	31	16.4	6	13.3	
Yes	158	83.6	39	86.7	0.778

*BMT = Bone Marrow Transplantation; NR = Not Reported

A significant association (p = 0.024) was found for individuals over 50 years old with probable, possible or proven IA results (Table 3). Six (35.3%) of these patients presented some type of neutropenia for confirmed IA diagnosis and 10 (66.7%) for the probable diagnosis. Fourteen (82.4%) of these patients were using antifungal drugs and had a proven diagnosis and 15 (88.2%) of the 17 patients, with a proven diagnosis, used an antibiotic and / or antiviral.

Table 3. Variables associated with the diagnosis of Invasive Aspergillosis according to European Organization for Research and Treatment of Cancer/ Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group, of suspected patients at the Araújo Jorge Hospital in Goiânia, Goiás, Brazil, from 2013 to 2015

Variables	Diagnosis of Invasive Aspergillosis								p-value
	Negative (n=190)		Possible (n=12)		Probable (n=15)		Proven (n=17)		
	n	f (%)	n	f (%)	n	f (%)	n	f (%)	
Gender									
Female	88	46.3	5	41.7	3	20.0	8	47.1	
Male	102	53.7	7	58.3	12	80.0	9	52.9	0.244
Age (years)									
≤ 20	18	9.5	3	25.0	3	20.0	7	41.2	
21 a 50	99	52.1	3	25.0	5	33.3	5	29.4	
> 50	73	38.4	6	50.0	7	46.7	5	29.4	0.024
BMT									
Alogenic	33	17.4	2	16.7	2	13.3	4	23.5	
Autologous	81	42.6	2	16.7	3	20.0	5	29.4	
No	76	40.0	8	66.7	10	66.7	8	47.1	0.224
Bilirubin									
≤ 1.0 mg/dL	92	48.4	4	33.3	5	33.3	8	47.1	
> 1.0 mg/dL	44	23.2	4	33.3	6	40.0	3	17.6	0.389
NR	54	28.4	4	33.3	4	26.7	6	35.3	
Neuropenia									
Light (>1000)	11	5.8	2	16.7	0	0.0	0	0.0	
Moderate (500-1000)	14	7.4	0	0.0	1	6.7	1	5.9	
Severe (<500)	64	33.7	2	16.7	9	60.0	5	29.4	
No neutropenia	101	53.2	8	66.7	5	33.3	11	64.7	0.253
Antifungal									
No	50	26.3	2	16.7	4	26.7	3	17.6	
Yes	140	73.7	10	83.3	11	73.3	14	82.4	0.766
Antibiotic + Antiviral									
No	32	16.8	2	16.7	1	6.7	2	11.8	
Yes	158	83.2	10	83.3	14	93.3	15	88.2	0.698
Death									
No	143	75.3	7	58.3	4	26.7	6	35.3	
Yes	41	24.7	5	41.7	11	73.3	11	64.7	<0.0001

Considering the total number of participants, 31.6% of the patients died. Patients with negative GM had a mortality rate of 24.7%. There was a significant association between patients who developed the disease and the mortality rate. Of the 44 patients who were diagnosed with IA, 27 died (61.3%, p <0.0001).

DISCUSSION

Invasive aspergillosis increased dramatically in the 1980s and 1990s in the United States and Europe according to autopsy reports performed during those periods (Zhang et al., 2015). Regarding patient age, a similar frequency was noted between men and women in the population in this study, with men being a little more frequent. The average age was 43.7 years. A similar result was found in the study conducted by Bhayat et al. (2010), who reported that the frequency according to sex was close, with males presenting a 5% higher frequency.

IA has traditionally been associated with immunocompromised patients, but its incidence in immunocompetent patients is increasing. This is according to a systematic retrospective review of 10 cases of IA performed in India from 2013 to 2015 due to the widespread use of immunosuppressive treatments, steroids and the frequent and indiscriminate use of antibiotics (Shah et al., 2017).

According to Garcia-Vidal et al. (2008), and Ok et al. (2011), the vulnerability to IA in transplanted patients can be considered multifactorial, covering both clinical risk factors and advanced age, as well as biological risk factors, such as deficiency in multiple cell lines and iron overload. Some of this information was noted in our study, which found statistically significant results (p = 0.024) for the association of patients older than 50, with IA diagnosis.

The mortality rate in patients developing IA may exceed 90%, being considered the main cause of morbidity and mortality with hematological diseases. In our study, 31.6% of the patients died. The association between disease development and mortality rate was extremely significant (61.3%, p <0.0001). Patient survival, according to studies, is closely associated with early diagnosis and immediate therapy (Badiee & Alborzi, 2010; Pfeiffer et al., 2006; Sherif & Segal, 2010; Xavier et al., 2011).

However, according to a systematic review recently performed (Rotjanapan et al., 2018), the survival of IA patients has increased in recent years due to advances in diagnosis and the release of newer antifungal drugs. Despite these advances, much still has to be improved (attributable mortality is 42-64%), especially in patients who have had allogeneic BMT, are of advanced age or in cases of extrapulmonary involvement. In patients who underwent BMT, the disease occurs in 15.1% of those who had allogeneic-type BMT and 2.0% in those who had autologous BMT (Badiee & Alborzi, 2010).

Analysis of the studied population showed that the frequency of patients who underwent allogenic BMT (17.9%) was lower than the frequency of those who had autologous BMT (38.9%). Thus according to these indices, the patients studied presented a lower probability of developing IA, considering only the type of transplantation performed. This situation can be confirmed when BMT data and the type of transplantation performed is associated with IA diagnosis, where 81 of the 91 patients who underwent autologous BMT presented negative IA results.

According to the data found in this study, 47.0% of the patients examined presented some degree of neutropenia, 34.2% of which had severe neutropenia. In contrast to the high rates of neutropenia found, Garcia-Vidal et al. (2008) reported that lymphopenia, neutropenia and / or monocytopenia were noted as strong risk factors for IA. Although these cytopenias were consistently identified in the vast majority of patients suffering from the disease, antifungal defenses involve more than death of neutrophils, where, for example, *Aspergillus* spp. had been recognized as fundamental in the regulation of pulmonary inflammation.

According to Pfeiffer et al. (2006), the population with the highest risk of developing IA is that with prolonged neutropenia, HIV infection and hereditary immunodeficiency. In our study, two patients had virus infection, but neither of them developed IA. However due to the small number of patients with HIV, it was not possible to describe any correlation between the virus and IA in this study.

The qualitative results for GM detected by verified ELISA found that 18.8% tested positive for GM. Despite the fact that the detection of GM in the serum may present low sensitivity when compared to the dosage in bronchoalveolar lavage fluid (Zhang et al., 2015), the use of the ELISA test for GM detection in serum was very useful for IA surveillance in patients with hematological malignancies (DHM) or patients receiving hematological transplants. However, in cases of solid organ transplants, the test presented low sensitivity and specificity (Pfeiffer et al., 2006). Thus, the test, when evaluating the high frequency of patients in the present study with DHM (96.9%) and patients receiving haematological transplants (56.8%), aids in prognosis, diagnosis and preventive therapy, because these factors make the patient highly susceptible to aspergillosis and make the test crucial for monitoring patients.

In contrast, the detection of serum GM may be influenced by the antifungal treatment administered and false positive results occurring in patients who received antibiotics from fungi (piperacillin-tazobactam and amoxiline with clavulanic acid), beta-lactam antibiotics, intravenous fluids containing gluconate, patients submitted to cytotoxic agents, autoreactive antibodies in cases of graft versus host disease, transfusions, sample contamination and infections caused by other fungi (*Penicillium, Paecilomyces* and *Histoplasma capsulatum*) (Rotjanapan et al., 2018; Sherif & Segal, 2010; Sun et al., 2016).

Due to this, the possibility of false positive cases in the population of this study should not be ruled out, since at the time of the GM examination, approximately 84.2% of the patients involved in the research had recently used or were using some antibiotic types and 74.8% used antifungals. Additionally, statistically the association of positive results for GM and the percentage of patients who used some antibiotic and / or antiviral drugs found that 39 patients of the 46 who presented positive results used these drugs, thus reinforcing the possibility of false-positive cases.

Jaundiced patients may also present false-positive results, due to the interference of the bilirubin pigment in the optical reading of the GM ELISA test (Xavier et al., 2011). In our study, the serum bilirubin indices associated with the quantitative galactomannan result was $p = 0.062$. There was, therefore, a biased outcome for such an association.

A study conducted in Turkey investigated the performance of a kit *Aspergillus* lateral flow (LFD) as a rapid care test to diagnose IA. This test is an immunoassay that is based on the detection of the *Aspergillus* antigen released during hyphae invasion by IgG monoclonal antibodies. Although there is no need for specific equipment to perform the test and the result is available in 15 minutes, the sensitivity for cutting-edge testing is low and commercialization is not yet available (Bassetti et al., 2017; Metan et al., 2017).

Voriconazole, or isavuconazole, second generation of triazoles can be used as the treatment of choice for IA; and for cases with low therapeutic response to this triazole, an alternative use of lipid formulations of amphotericin B is recommended, as it is less nephrotoxic (Cadena et al., 2016). In the present study, the majority of patients (74.8%) used some antifungal, where according to their medical records the drug of choice was voriconazole, amphotericin B lipid or both. A review describing the use of a drug called isavuconazole, which is a second generation triazole presenting activity against a broad spectrum of clinically important fungi, reported that this drug when compared to voriconazole, showed fewer adverse effects than voriconazole. The authors further report that there are several features that make isavuconazole a novel treatment option for IA, including predictable pharmacokinetics, excellent bioavailability, no oral side effect and potential usefulness in patients with renal insufficiency, due to the absence of cyclodextrin, a component in the composition of voriconazole, which makes it nephrotoxic (Chen et al., 2017).

In summary, when considering the high mortality rate caused by the development of IA and the fact that early therapy significantly improves patient prognosis, the results found in this study allow us to suggest that the detection of GM performed as a follow-up, in patients with high risk of developing the disease as a preventive form, may be considered an effective method to assist in the identification of IA.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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