Original Research Article

Role of *Aspergillus* Galactomannan Antigen Test for the Screening of Invasive Aspergillosis in patients with Hematological Malignancies

Running title: Screening of Invasive Aspergillosis in Hematological Malignancies.

ABSTRACT

Background: Invasive aspergillosis (IA) is a leading cause of death among immunocompromised patients, particularly those with hematological malignancies. The use of galactomannan (GM) antigen as a biological marker for screening of IA in high-risk patients is attractive and non-invasive tool that detect evidence of IA prior to the appearance of clinical manifestations.

Objectives: The aim of this study was to compare the diagnostic value of the conventional blood culture technique to the serological detection of GM antigen using ELISA for screening of IA in neutropenic patients with hematological malignancies.

Methods: Forty patients with haematological malignancies from those admitted to the Clinical Oncology Department of Menoufia University Hospitals (MUH) were enrolled and classified to have either proven (5/40; 12.5%), probable (10/40; 25%) or possible (25/40;62.5%) invasive aspergillosis based on the clinical criteria provided by the European Organization for research and treatment of Cancer (EORTC) and Mycoses Study Group (MSG). Blood samples were collected from all participants and subjected to conventional blood culture for isolation and identification of *Aspergillus* spp. ELISA technique was applied for serological detection of GM antigen in the patients' serum samples.

Results: The sensitivity, specificity, PPV and NPV of GM antigen ELISA testing were 100%, 74%, 36% and 100% respectively for both proven and probable cases. On applying the principle of test in series (the patient is positive if positive in both culture and the GM) , the results were improved to 100% sensitivity, 100 % specificity, 100% PPV, 100% NPV and 100% all over accuracy. Galactomannan antigen testing proved excellent sensitivity compared to other clinical features and radiological criteria for diagnosis of probable aspergillosis (100% versus 80%) and proved to be a good negative test.

Conclusion: With conjunction of clinical and radiological signs, *Aspergillus* galactomannan test can assist physicians in the of diagnosis of IA in patients with hematological malignancies to allow initiation of effective antifungal therapy which is ultimately important in high-risk populations

Keywords: Invasive aspergillosis, galactomannan antigen, blood culture, hematological malignancies.

1. INTRODUCTION:

Invasive fungal infections (IFI) are associated with high morbidity and mortality in immunocompromised patients with hematological malignancies **[1].** Several factors are incriminated in increasing frequency of IFI among these patients like dose-intensive regimens, mucosal damage (due to chemotherapy) and widespread use of broad-spectrum antibiotics **[2].**

Diagnosis of IFI in patients with hematologic malignancies remains difficult and challenging since the clinical symptoms are often difficult to distinguish from symptoms of bacterial or viral infections. Moreover, conventional diagnostic methods are associated with shortcomings due to lack of sensitivity of conventional culture methods. Non-culture-based techniques, such as detection of DNA by PCR or measurements of fungal biomarkers in blood or respiratory samples, are important adjunctive tools **[3]** & **[4].**

Invasive aspergillosis (IA) is a potentially life-threatening infection, caused mainly by different *Aspergillus* spp. of which *A. fumigatus* is the most prevalent. In serious cases, *A. fumigatus* may spread from the lung to other organs, resulting in haematogenously disseminated fungal disease **[5].**

Galactomannan (GM) is a polysaccharide antigen that exists primarily in the cell walls of *Aspergillus* species. GM may be released into the blood and other body fluids even in the early stages of *Aspergillus* invasion and the presence of this antigen can be sustained for 1 to 8 weeks. Therefore, detection of the GM antigen level via enzyme linked immunosorbent assay (ELISA) can be useful in making an early diagnosis of invasive aspergillosis **[6].**

Currently, serum GM detection is considered a microbiological diagnostic criterion for fungus infection in neutropenic patients, according to the guidelines of 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) [7]. Detection of in GM antigen in serum and recently in other body fluids provides easily performed assays for mycological supportive criteria that in conjunction with imaging scan findings can be used to support the diagnosis of probable IFI [8].

Because early diagnosis of IFI became necessary to avoid delaying in treatment initiation which is associated with an increased mortality [9], here came the aim of this research which tried to findout the validity of serological detection of *Aspergillus* galactomannan antigen in relation to the conventional culture methods for the screening of invasive aspergillosis in neutropenic patients with hematological malignancies at Menoufia University Hospitals.

2. PATIENTS AND METHODS:

This study was conducted from March to June 2019 at the Department of Medical Microbiology and Immunology, Faculty of Medicine, Menoufia University in collaboration with Clinical Oncology Department and Clinical Microbiology and Immunology Department of the National Liver Institute.

2.1 Study population:

This study included 40 patients (14 females and 26 males) with mean age of 52.32 ± 10.05 years, with hematological malignancies (lymphomas and leukemias) who were admitted to the Clinical Oncology Department with suspected IFI.

2.2 Collected data

All patients were subjected to full history taking including name, age, sex, type of malignancy, type, dose and duration of chemotherapy (immunosuppressive), history of antibiotics and or antifungals received. General examination was also performed for body temperature recording, examination of oral cavity for ulcers or thrush, looking for nasal ulcers or sinusitis, chest, abdominal and CNS examination. All selected patients had no previous history of *Aspergillus* galactomannan antigen testing.

Routine laboratory data including baseline complete blood picture (CBC), liver and kidney function, radiological findings as chest X-ray and CT scan were collected from patients' files, for detection of internal organ involvement. Blood samples for fungal and bacterial culture, swabs were taken from suspected sites of infection (when possible) for fungal analysis.

2.3 Specimen collection

Blood samples were collected by withdrawing 10 ml of venous blood from each adult patient and 2–5 ml from infants and children under strict aseptic conditions. The sample was divided as follows; 0.5 ml in infants to 1.5ml in adult; were inoculated into culture bottles in BACT/ALERT 3D system (BioMerieux**)** for five days. Upon appearance of any alarm indicative of possible growth in the blood culture bottle, subculture were made on Sabaroud's Dextrose media (BD Bioxon) at 37°C for 2-5 days for detection of *Aspergillus* spp. and other fungal infections in the patient's blood.

2.4 Identification of *Aspergillus* spp.: Microscopic examination of Gram stained film of *Aspergillus* spp. showed Gram positive septate hyphae with dichotomous branching. *A. fumigatus* produced a fluffy and granular colony and exhibited blue-green powdery appearance. *A. flavus* produced a yellow-green colony. *A.niger* began as a yellow colony that soon became black, dotted surface as conidia are produced. With age the colony became jet black **[10].**

2.5 Identification of *Candida* spp.: Up to species level was performed by culture onto Brilliance™ *Candida* Agar [Oxoid Chromogenic Candida Agar (OCCA) CODE: CM1002; England]; the inoculated plates were inspected for the growth of *Candida* spp. at 24, 48 and 72 hrs. The grown colonies were identified according to their expected reactions on the plate **[11].**

2.6 Identification of the isolated bacterial spp.: Also, subcultures were made on nutrient, blood, mannitol salt and MacConkey's agars and incubated aerobically at 37°C for 24-48 h for identification of possible mixed bacterial infection. The resultant colonies were subjected to further morphological and biochemical reactions to identify different bacterial species according to the standard microbiological methods **[12].**

2.7 Serological detection of *Aspergillus* galactomannan antigen

Aspergillus GM antigen was detected in patient's serum via enzyme-linked immunosorbent assay (PLATELIA™ ASPERGILLUS Ag BIO-RAD USA) according to the manufacture's instructions; 2 ml of whole blood were allowed to clot and serum was separated, divided into aliquots and stored at 2 °C to 8°C for up to 48 h or frozen at −80°C until testing for GM antigen. To calculate the mean cut-off control OD, the OD values for each cut-off control were added together and the result was divided by 2 and the index of each test sample was calculated by dividing the OD of the sample by the mean cut-off control OD, the results were interpreted as index (I): serum with index less than < 0.50 and index ≥ 0.50 were respectively considered to be negative and positive for GM antigen in both male and female patients.

Different specimens of suspected clinical sites were also obtained from 10 patients (pus, oral swaps, vaginal swaps and sputum) and inoculated onto nutrient, blood,

mannitol salt and MacConkey's agars and incubated aerobically at 37°C for 24-48 h; for identification of bacterial infection. The resultant colonies were subjected to further morphological and biochemical reactions to identify different bacterial species according to the standard microbiological methods. Specimens were inoculated also onto Sabaroud's Dextrose agar at 37°C (BD Bioxon) for detection the presence or absence of fungal infection (*Candida* spp. and *Aspergillus* spp.) **[12].**

The culture results were identified, total period of patients' admission during their illness was identified, and also patient's fate and the average cost for admission per patient were calculated.

The study populations were categorized into three groups {as per the international consensus of the IFI cooperative group of the European Organization for research and treatment of Cancer (EORTC) and Mycoses Study Group (MSG)} **[7].** Group I involved 5 patients with aspergillosis (confirmed by positive blood culture). Group II involved 10 patients with probable aspergillosis. Patients in this group were those who had a host factor (like recent history of neutropenia and uncontrolled fever despite use of broad-spectrum antibiotics), a clinical feature (the presence of 1 of the 3 signs on CT chest e.g. dense, well circumscribed lesions (s) with or without a halo sign, air-crescent sign or cavity, tracheobronchial ulceration, nodule, pseudo-membrane, plaque, or eschar seen on bronchoscope analysis, sino-nasal infection, Imaging showing sinusitis plus, acute localized pain or nasal ulcer with black eschar, CNS infection with focal lesions and/or meningeal enhancement on MRI or CT) in addition to a mycological evidence in the form of positive microscopy or culture from samples such as sputum, bronchoalveolar lavage or sinus aspirate. *Aspergillus* GM antigen test was excluded as criterion of probable fungal infection. Group III involved 25 patients had host and clinical factors without mycological evidence

2.8 Statistical analysis:

Data were analyzed by SPSS statistical package version 23 (SPSS Inc. Released 2015. IBM SPSS statistics for windows, version 23.0, Armnok, NY: IBM Corp.). ANOVA test was used for comparison of quantitative variables between more than two groups of normally distributed data. Chi-square test ($\chi 2$) was used to study association between qualitative variables. Whenever any of the expected cells were less than five, Fischer's Exact test was used. Two sided P- value of < 0.05 was considered statistically significant.

3. RESULTS:

The mean age of the participants was 52.32±10.05 years. Twenty percent (8/40) of the patients had chronic lymphocytic leukemia (CLL), 10% (4/40) had acute myeloid leukemia (AML), 57.5% (23/40) had non-Hodgkin's lymphoma (NHL), 10% (4/40) had acute lymphocytic leukemia (ALL) and 2.5% (1/40) had relapsed Hodgkin's diseases (HD).

Bacterial blood culture was positive in 15 patients only; representing 37.5% of all the included cases and the most commonly isolated bacterial species were *Klebsiella pneumoniae.* Fungaemia was present in 9 cases (22.5%); four cases were *Candida albicans* (10%) and 5 cases were aspergillosis (12.5%) (Table1).

The prevalence of invasive fungal infection (proven and probable) was 22.5% (9/40 patients), among them 5 patients only had positive culture for invasive aspergillosis (12.5%) as shown in (figure1). Among them, *Aspergillus* GM antigen was positive in 100% (5/5), 80% (8/10), and 4% (1/25) of patients with proven, probable and possible fungal infection respectively (figure 1).

In relation to the standard blood culture results, the GM had 100% sensitivity, 74.0% specificity, 36.0% PPV, 100.0% NPV and 78.0% over all accuracy among the whole studied groups.

On applying the concept of test in parallel (the patient is positive if positive in the culture or the GM), no more diagnostic value were added to the results of GM validity (100% sensitivity, 74.0% specificity, 36.0% PPV, 100.0% NPV and 78.0% all over accuracy). While on applying the principle of test in series (the patient is positive if positive in both culture and the GM), the results were improved to 100% sensitivity, 100.0% specificity, 100.0% PPV, 100.0% NPV and 100.0% all over accuracy (Table 2 and 3).

Comparison between the studied patients' groups according to their clinical features showed no significant difference regarding their age, gender or initial diagnosis. Bacterial culture positivity was more among cases with possible fungal infection (Table 4).

The prognosis of patients with invasive aspergillosis was analysed in table 5 where the mortality rate among patients with invasive aspergillosis diagnosed by GM antigen detection reached 50% while for patients with invasive aspergillosis diagnosed by blood culture detection, it was 80%.

4. DISCUSSION:

Invasive aspergillosis (IA) represents a serious threat in patients with depressed immune system. Patients diagnosed with hematologic malignancies and chemotherapy-induced neutropenia or hematopoietic stem cell transplantations (HSCT) are at highest risk **[13].**

The current diagnostic limitations require improvement for detection of fungal pathogens by defining the optimal use of biomarkers and clinical samples. Being invasive, histopathological examination not done in most patients and proven cases are diagnosed based on positive blood culture for aspergillosis. Therefore, sensitive assays for accurate diagnosis of the fungal infection are needed **[14], [15].**

In the current study 40 patients with different types of hematological malignancies like lymphoma and leukemia were enrolled. Patients were classified to have either proven (5/40; 12.5%), probable (10/40; 25%) or possible (25/40; 62.5%) invasive fungal infection as per the European Organization for research and treatment of Cancer (EORTC) and Mycoses Study Group (MSG).

Among the studied population, the incidence of proven, probable and possible aspergillosis was 12.5%, 20% and 2.5% respectively. **Zhang et al [15]** reported that, among the studied population the incidence of proven aspergillosis was 0.8%, probable aspergillosis was 14.0% and possible aspergillosis was 7.4%. **Dos Santosa et al [16],** reported that overall incidence of invasive aspergillosis (proven and propable) detected by *Aspergillus* GM screening test in hospital in Brazil was 12.2%**,** and, **Penack et al., [17]** who found the incidence of proven, probable and possible IA was 6%, 2.5% and 15.5%, respectively. The previously reported data provide additional support for the importance and seriousness of the IA infection in this group of immunosuppressed patients.

PLATELIA™ Aspergillus kit for detection of GM antigen has been used in diagnosis of invasive Aspergillosis with the standard blood culture method; Considering true positives as the only results obtained for patients with proven and probable IA; the sensitivity, specificity, positive and negative predictive values of GM testing in parallel to the standard blood culture in diagnosing of proven Aspergillus infection were 100, 74, 36, 100 and 78 % respectively. And this was comparable to that found by **Foy et al. [18]** who found the sensitivity of both GM antigen test and blood culture were the same (100%), and **Pfeiffer et al. [19]** who found the sensitivity and specificity of GM testing in proven cases of invasive Aspergillosis were 71% and 89% respectively. On the other hand, **Penack et al. [17]** reported that GM testing had excellent specificity (100%) and sensitivity was relatively low (40%) at onset of fever, but increased to 94.7% after 6 days of fever. And in study conducted by **Ahmad, et al. [2]** PPV and NPV were 79 % and 83% respectively which is lower than our results this may be due to different study design as we directly compare between blood culture and GM ELISA test.

The sensitivity of galactomannan assay for IA has been variably reported among studies. This variability in the assay may be related in part to the hosts, their exposure to antifungal agents and cut-off value of a positive GM result. The excellent sensitivity and negative predictive value make this approach suitable for clinical decision making.

Among probable cases; the sensitivity, specificity, PPN and NPV were as follow 100%, 74%, 36% and 100% respectively, these values were close to that reported by **Tănase et al., [20]** who found the Sensitivity (85%), Specificity (91%), PPV (46%) and NPV (99%). On the other hand,**Pfeiffer et al. [19]** reported the sensitivity and specificity of GM testing in diagnosis of probable cases of invasive Aspergillosis were 61% and 93% respectively.

The excellent sensitivity of GM antigen test compared to other features to diagnose probable fungal infection (100% versus 80%) also with high NPV it is a good negative test.

However, both the test and the other features for probable fungal infection are troubled with its low PPV. Making combination of the test with other features for diagnosing patients with probable infection considered and recommended by **De Pauw et al [7]** and **Tănase et al. [20]**

Many false-positive test results are reported with the GM assay, and this because some number of β-lactam antibiotics, including Penicillium, piperacillin/tazobactam, amoxicillin/clavulanate, and ampicillin have yielded positive Platelia EIA results. False positive results are reported more frequently in children and it was suggested that GM in present in milk or protein-rich nutrients and this is the cause of false-positive results in children. This was supported by the high false positive rate (83%) reported in newborn babies by **Mennink-Kersten et al. [8].** Other causes related to false positive results including: infection with organisms that share cross-reacting antigens with Aspergillus, reduced renal clearance, patients undergoing liver transplantation for autoimmune liver disease, patients undergoing cyclophosphamide treatment, patients undergoing lung transplantation for cystic fibrosis and chronic obstructive pulmonary disease**[8].**

Also, false- negative test results are reported with the GM assay, The main reason for it is exposure to antifungal agents but, other causes are also important including: inappropriate diagnostic criteria for IA, inadequate frequency of galactomannan testing, patients with non or minimally invasive manifestation of aspergillosis and low volume of sampling or long-term storage of samples **[8].**

Bad prognosis are reported in patients infected with invasive aspergillosis\, our results showed that high GM indices in serum of patients with IA positively correlated with fatal outcome, as 50% of patients infected with IA died and; this is also reported with **Penack et al., [17], Sheppard et al., [21]** and **Maertens et al. [22].**

Among the three studied groups; there was no relation between category of invasive fungal infection and their diagnosis, This was against results of **Racil et al [23]** who reported that proven and probable fungal infection was more prevalent among leukemic patients and this difference can be related to difference in sample composition as most of our patients were diagnosed with lymphoma.

The incidence of bacterial culture positivity was more among cases of possible fungal infection (40%) and this explains the uncontrolled fever among this group of immune compromised patients.

In the diagnosis of invasive aspergillosis, the galactomannan ELISA was positive before other microbiologic evidence of aspergillosis was available (within 48 to 72 hours). While the average period for Aspergillus culture was 3 to 10 days.

The cost per patient of Aspergillus galactomannan antigen test was cheaper(133 Egyptian pounds)in comparison with fungal blood culture (200 Egyptian pounds).Being a good negative, rapid and affordable test, we recommend Aspergillus galactomannan antigen test to be done routinely for any patient suspected of having fungal infection.

5. CONCLUSION AND RECOMMENDATION:

The authors recommend checking serum GM antigen assay as a screening test for hematologic malignancies. The results of GM antigen assays, if positive, can be used to support the diagnosis of probable fungal infection in patients who also meet the host factors and clinical criteria and should encourage the physician to start empiric antifungal therapy. Negative results of GM antigen assays in patients with persistent neutropenic fever without clinical or radiologic findings are more helpful in supporting the absence of IA and avoiding empiric anti-mold treatment. Further studies with attention to the impact of antifungal therapy, rigorous assessment of false-positive test results, and assessment of the utility of the test under non-surveillance conditions are needed.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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Table 1. Demographic characteristic of studied patients

	No. (%)
Age (mean ±SD, range)	52.32 ± 10.05, 29-72
Gender	
Male	26 (65.0)
Female	14 (35.0)
Diagnosis	
CLL	8 (20.0)
AML	4 (10.0)
NHL	23 (57.5)
ALL	4 (10.0)
Relapsed HD	1 (2.5)
Bacterial culture	
Positive	15 (37.5)
Negative	25 (62.5)
Bacterial species	
Streptococcus	1 (2.5) (6.7)
Klebsiella pneumoniae	10 (25.0) (66.7)
E coli	3 (7.5) (20.0)
Mixed	1 (2.5) (6.7)

Fungal blood culture Positive Negative	 9 (22.5) 31 (77.5)
Fungal species *Candida albicans* Aspergillosis	 4 (10.0) (44.4) 5 (12.5) (55.6)

Fig.1. The percent's of invasive aspergillosis by both blood culture technique and galactomannan antigen detection techniques in the studied groups.

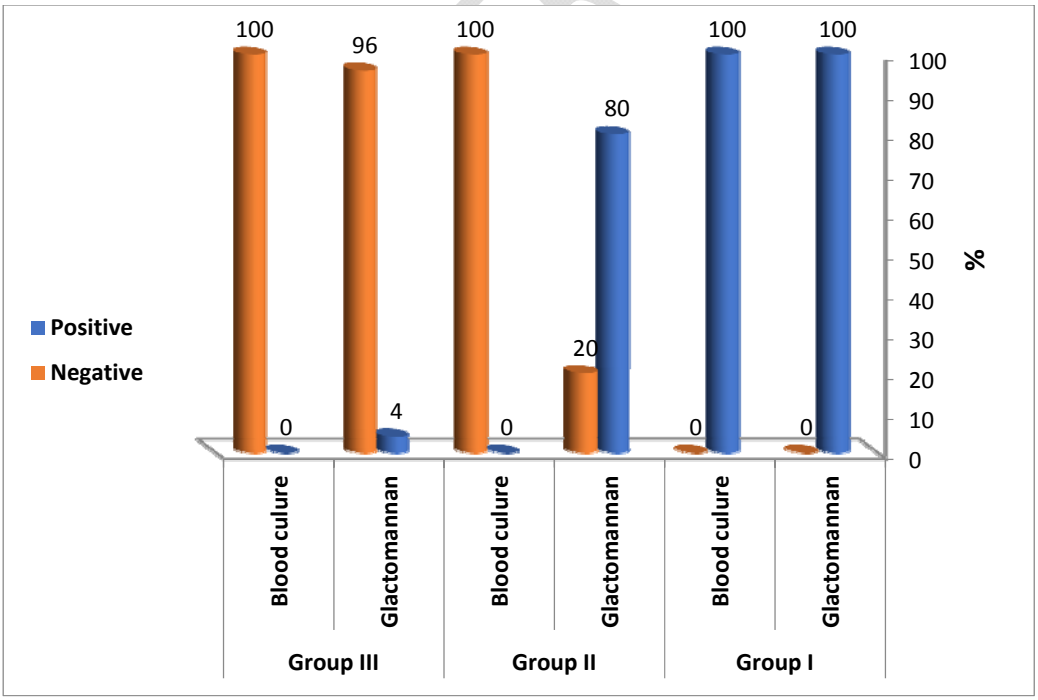

Table 2. Validity of Aspergillusgalactomannan test in parallel to the standard blood culture in diagnosis of proven Aspergillus infection:

	Sensitivity	Specificity	PPV	NPV	Accuracy

Aspergillusgalactomannan antigen test	100%	74%	36%	100%	78%
Aspergillus Culture	100%	100%	100%	100%	100%

Table 3. Validity of Aspergillusgalactomannan test in diagnosing probable cases in parallel to Clinical and Radiological features:

	Sensitivity	Specificity	PPV	NPV	Accuracy
Aspergillusgalactomannan antigen test	100%	74%	36%	100%	78%
ClinicalandRadiological features	80%	74%	31%	96%	75%

Table 4. comparison of clinical features amongthe studiedpatients' groups

	Proven (n=5) No. (%)	**Probable (n=10) No. (%)**	**Possible (n=25) No. (%)**	**P value**
Age (mean ±SD, range)	54.20 ± 9.85	49.00 ± 9.41	53.28 ± 10.41	0.55
Gender Male Female	 4 (80.0) 1 (20.0)	 5 (50.0) 5 (50.0)	 17 (68.0) 8 (32.0)	 0.55
Underlying haematological diseases CLL AML NHL ALL Relapsed HD	 2(40.0) 0 (0.0) 2 (40.0) 1 (20.0) 0 (0.0)	 2(20.0) 2 (20.0) 5 (50.0) 1 (10.0) 0 (0.0)	 4 (16.0) 2 (8.0) 16 (64.0) 2 (8.0) 1 (4.0)	 0.754
Bacterial culture Positive Negative	 5 (100.0) 0 (0.0)	 0 (0.0) 10 (100.0)	 10 (40.0) 15 (60.0)	 <0.001
Body temperature High grade fever Normal body temperature	 5 (100.0) 0 (0.0)	 10 (100.0) 0 (0.0)	 25 (100.0) 0 (0.0)	 ------
Neutropenia (< 500) Present Absent	 5 (100.0) 0 (0.0)	 10 (100.0) 0 (0.0)	 25 (100.0) 0 (0.0)	 ------
Antibiotic administration Yes No	 5 (100.0) 0 (0.0)	 10 (100.0) 0 (0.0)	 25 (100.0) 0 (0.0)	 ------
Radiological finding				

Yes No	**2 (40.0)** **3 (60.0)**	**6 (60.0)** **4 (40.0)**	**0 (0.0)** **25 (100.0)**	**<0.001**

Table 5. Prognosis of Hematological Malignancies' Patients and patients with Invasive aspergillosis.

	Fate		**FE test**	**P value**
	Passed	**Died**		
Aspergillusgalactomannan antigen test **Neg** **Pos**	 22 (75.9) (84.6) 7 (24.1) (50.0)	 4 (36.4) (15.4) 7 (63.6) (50.0)	 5.46	 0.029
Aspergillus Culture **No** **Yes**	 28 (96.6) (80.0) 1 (3.4) (20.0)	 7 (63.6) (20.0) 4 (36.4) (80.0)	 7.90	 0.015