Diagnostic potential of nested PCR assay and galactomannan ELISA for invasive aspergillosis in pediatric patients with haematological malignancies in Menoufia University Hospitals, Egypt

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Abstract: Objective: The aim of this work is to detect the incidence of invasive aspergillosis (IA) among children with hematological malignancies, and to compare the diagnostic performances of galactomannan antigen test by ELISA kit, and nested-PCR for aspergillus DNA, in order to choose early and rapid method for diagnosis of IA in a trial to improve prognosis and reduce the use of antifungal drugs. **Background**: Pediatric patients with cancer, particularly those with hematologic malignancies, are at increased risk of developing invasive aspergillosis (IA), which is a major cause of morbidity and mortality in immunosuppressed patients. The diagnosis of IA is difficult and early diagnosis and initiation of antifungal therapy is important to achieve the best outcome. Obtaining representative sample material using invasive procedures may be difficult in pediatric settings. The detection of the GM antigen, a constituent of polysaccharide fungal cell wall and the detection of fungal DNA by a PCR method have been developed with the aim to quickly screen patients for IA. **Method**: We collected blood samples from 36 febrile neutropenic leukaemic children following chemotherapy and suspected to have IA, these samples were tested by GM ELISA and nested PCR in addition to blood culture for fungus. **Result**: The incidence rate of invasive aspergillosis in the patients was found to be(36.1%) by nested PCR. Two (5.5%), ten (27.8%), and twenty four (66.6%) of patients had proven, probable and possible IA respectively by EORTC / MSG definition of invasive fungal infections. Sixteen (44.4%) of cases were positive for galactomannan test (GM) and the galactomannan index of the patients ranged from 0 – 3.9, and mean ± SD was 1.12 ± 1.36. There was non-significant difference between negative and aspergillus infected patients by nested PCR regarding age, sex, type of leukemia and number of febrile attacks. There was highly significant difference in the mean value of temperature and hemoglobin level between positive and negative aspergillus patients by nested PCR (*P - value <0.001*). There was a statistic significant difference in the mean value of ANC between aspergillus infected patients and negative patients by PCR *(P-value <0.03*). The best cut off level of serum galactomannan index was 0.8, where sensitivity, specificity, PPV, NPV and of accuracy of galactomannan was (100%, 91.3%, 86.7%, 100%, 94.4%) respectively. Sensitivity, specificity, PPV, NPV of nested PCR was found to be 85.7%, 95.5%, 92.3%, 91.3% respectively. **Conclusion:** Galactomannan and nested-PCR tests, are with enough accuracy and reliability that can serve as noninvasive methods for the detection of IA in pediatric hematological patients.

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Key words: invasive aspergillosis, nested PCR, galactomannan, hematological malignancies

1. Introduction

Invasive fungal infections (IFI) are serious infections and often life threatening and remain an important cause of morbidity and mortality among immunocompromised patients. The most common invasive fungal infections include invasive candidiases, aspergillosis and cryptcoccosis ***(Penack et al., 2008)***.

The incidence of IFIhas increased significantly over the past two decades because of increasing use of aggressive chemotherapy, increasing number of stem cell transplantation, widespread use of antifungal prophylaxis and increasing number of organ transplant recipients ***(Walsh et al., 2008)***.

Invasive aspergillosis is caused by *Aspergillusfumigatus* and other *Aspergillus species*. Being an abundant component of inhaled air, this organism represent one of the leading causes of IFI-related morbidity and mortality ***(Choi et al., 2013)***.

Hematological malignancies in children may derive from either of the two major blood cell lineages; myeloid and lymphoid cell lines. Leukemia is the most common cancer in children and teens, accounting for almost 1 out of 3 cancers ***(Mushtag et al., 2013).***

About 3 out of 4 cases of childhood leukemia are ALL. This leukemia starts from the lymphoid cells in the bone marrow ***(Biondi et al., 2012).***

Diagnosis of invasive aspergillosis is difficult and challenging as clinical manifestations are non specific, thus, anti-fungals have been used empirically for decades in refractory neutropenic fever with high cost and toxicity ***(Dinleyici, 2011)***. Non invasive methods for early diagnosis are of great value. The detection of galactomannan (GM) antigen and detection of fungal DNA by polymerase chain reaction (PCR) method have been developed with the aim to quickly screen patients for invasive aspergillosis ***(Dinleyici, 2011)***.

Galactomannan (GM) is a polysaccharide consisting of a mannose backbone with galactose side group which is a component of the cell wall of *Aspergillusspp,* that is released into the blood stream by growing hyphae and germinating spores and conidia. The detection of galactomannan in serum is used to diagnose invasive aspergillosis in human through ELISA assay. ***(Jha et al., 2013)***.

In the last years, detection of *Aspergillus* nucleic acid by PCR in serum and blood is another useful tool for the diagnosis of IA in pediatric patients, especially those on diets rich in dairy products or receiving piperacillin / tazobactam or amoxicillin / clavulanate antibiotic treatment which give false positive results with galactomannan test ***(Ribeiro et al., 2006)***.

Classification of IAInvasive aspergillosis was classified according to the EuropeanOrganisation for Research and Treatment of Cancer (EORTC)/Mycology Study Group (MSG) criteria. Proof of infection (‘proven’ IA) was based on: histopathological orcytopathological evidence of hyphae with evidence of associated tissue damage, or a positive culture from a normal lysterile and clinically or radiologically abnormal site consistent with infection. ‘Probable’ IA required at least one host risk factor, one microbiological criterion, and one major (or two minor) clinical criteria. ‘Possible’ I Arequired at least one host factor, plus one microbiological criterion or one major (or two minor) clinical criteria ***(Ascioglu et al, 2002).***

2. Subjects and Methods

I) Subjects

The present study was carried out in the period between May 2014 to October 2015. It was done at clinical pathology department – Faculty of Medicine – Menoufiya University. The patients were selected from pediatric hematology and oncology department, Menoufiya University Hospitals.

Patients criteria

This study was conducted on 36 febrile neutropenicleukaemic children following chemotherapy and suspected to have IFIs.

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

All patients had fever (two or more axillary temperature readings above 38^{o}C in a 24 hour period or a single reading of 38.5^{o}C or higher), neutropenia (with absolute neutrophil count of 500/mm^{3} or lower during febrile period) and not responding to broad spectrum antibiotics for at least 7 days.

After the consent from the patients parents, **all the studied cases were subjected to the following:**

A) **Complete history taking**: as age, sex, type of leukemia, history of chemotherapy, history of antibiotics and/or antifungal received.

B) **General examination:** including body temperature, chest, abdominal and CNS examination, ENT examination for nasal ulcers with black eschar.

C**) Radiological examination: As** chest x-ray and computed tomography (CT) for chest, CNS and sinuses. Chest CT scans was performed when aspergillosis was suspected or X- ray showing air crescent. When chest CT showed a positive sign of aspergillosis (dense well-circumscribed lesions with or without a halo sign, air-crescent sign and cavity), sequential chest CT were repeated till 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

D) The following laboratory investigations were done:

1- CBC (Hb, WBC, ANC and PLT counts).

2- Histopathological and cytological examinations of biopsies.

3-Microbiological assessment:

- Blood culture for fungus.
- Culture of BAL, sputum, biopsies

4-Galactomannan antigen test by ELISA.

5-Nested PCR for detection of aspergillus DNA.

II- Methods

A) Sample collection and preparation:

-Clinical samples (including sputum, cerebrospinal fluid, bronchoalveolar- lavage or bronchial washing, sinus aspirate and biopsy) from those with positive clinical signs were tested for aspergillus infections by microbiological methods.

****-Blood samples**

8-10 milliliters of venous blood were withdrawn from each patient under complete aseptic condition by venipuncture. The sample was divided as follows:

a-Blood culture bottle: 0.5 – 1.5 ml were added to blood culture bottle for fungal culture.

b-Tube A: 2 ml of whole blood were dispensed into K3- EDTA vacutainer tube for CBC.

c-Tube B: 3 ml of whole blood were dispensed into sterile EDTA vacutainer collection tube and stored at -20°C until performance of nested PCR.

d-Tube C: 2 ml of whole blood was allowed to clot and serum was separated, divided into aliquots and stored at 2°C to 8°C for up to 48 hours or frozen at -20°C until testing for galactomannan antigen test.

B) Analytical methods:

1) Blood culture for fungus

By BACTEC BD for fungal (BACTEC™ Myco/F Lytic Culture Vials). Subcultures on sabaroud's dextrose agar and the blood agar plates which incubated at 37°C for detection of candida and bacterial isolates. Another sabaroud's dextrose agar inoculated and left at room temperature (22°C) for up to two weeks for detection of filamentous fungal growth.

Identification:

Identification of Aspergillus:

Most *Aspergillus sp.* grow relatively rapidly (typically within 48 hours). Extending incubation time from 2 to 5 days will increase the number of positive cultures.

Culture characteristics colony morphology:

*A.fumigatus*is a rapidly growing mold (2 to 6 days) that produces a fluffy and granular colony, most often exhibit the blue-green powdery appearance.

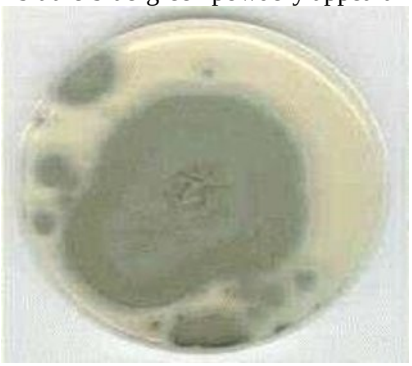

Fig (1): Aspergillus fumigates on SDA agar

Microscopic examination of gram stained film: stained gram positive with septate hyphae that exhibit dichotomous branching. The hyphae may not take the dye makes the identification difficult.

N.B *A.fumigatus*: gram positive septate hyphae, septation is observed and no constriction is seen.

2) Serum galactomannon antigen determination (ELISA): PLATEIA™ ASPERGILLUS AG (96 test) BIO RAD: Product No. 627942

The Platelia™ Aspergillus Ag is a one-stage immunoenzymatic sandwich microplate assay which detects galactomann in human serum. The assay used rat EBA-2 monoclonal antibodies, which are directed against *Aspergillus* galactomannan.

N.B Serum samples are heat-treated in the presence of EDTA in order to dissociate immune complexes and to precipitate proteins that could possibly interfere with the test.

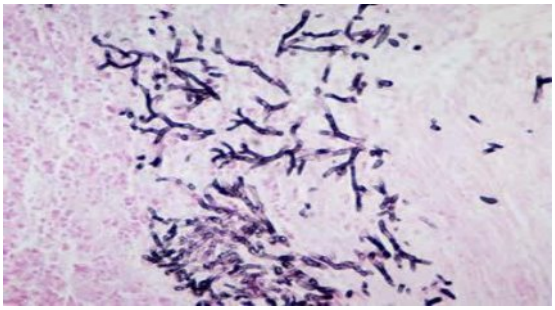

Fig (2): Gram stained film of aspergillus

3) Nested PCR for detection of aspergillus DNA.

a) DNA extraction by Gene JET DNA purification kit Thermo Scientific, Fermentas, UE). # K0721

1-Sample preparation: 500 µl EDTA anti-coagulated blood were mixed with 1500 µl red- cell lysis buffer (RCLB) and were incubated on ice for 10-15 minutes.

N.B: RCLB: (10 mM Tris PH 7.6, 5 mM $MgCl_2$, 10 mM NaCl).

2-The mixture was centrifuged at 3000 rpm for 10 min and the supernatant was discarded.

3-Steps 1 and 2 were repeated, then step 4 is continued.

4-The cell pellet was re-suspended in 1 ml white cell lysis buffer (WCLB). (WCLB = RCLB containing 200 ug/ml proteinase K) and was incubated at 65°C for 45 min.

5-Then centrifugation was done at 5000 rpm for 10 min and the supernatant was discarded.

6-Optional: 200 µl of NAOH was added. Then was covered with mineral oil and was incubated at 95°C for 10 min. the supernatant was discarded.

7-500 µl lyticase solution were added and incubated at 37°C for 30 min to produce spheroplasts.

N.B Lyticase solution: (10 u/ml lyticase, 50 mM tris pH 7.5, 10 mM EDTA, 28 mM B- mercaptomethanol).

8-Centrifugation was done at full speed for 10 min and the supernatant is discarded.

9-Tissue protocol is then continued with Mammalian tissue Genomic DNA Purification protocol.

b) DNA Amplification For the nested two-step PCR technique, two pairs of oligo-nucleotide primers (AFU 7S and AFU 7AS for the first step and AFU 5S and AFU 5AS for the second step) derived from sequences of the aspergillus fumigatus 18S rRNA gene (GenBank accession no. AB008401) and specific for aspergillus species were used.

N.B Suspensions of blood with aspergillus fumigatus conidia (1 to 10^5 conidia/ ml) were used to

determine the limit of the assay, and each solution was used for DNA extraction and PCR.

Table (1): Primers used for amplicatin of AFU gene

Target	Primer	Sequence (5^{-} -3^{-})
AFU	AFU 7S-20	CGG CCC TTA AAT AG CCC G
	AFU 7AS – 20	GAC CGGG GTT TGA CCA ACT TT
	AFU 5S-20	AGG GC CAG CGA GTA CAT CAC CTTG
	AFU 5AS-20	GGG RGT RGT CGT TGC CAA CY CYC CTG A

Reaction mixture*:*

A) first round PCR

The total reaction volume for the first – round PCR amplification is 25 µl which consist of:

1- 5 µl of template DNA.

2- 12.5 µl of Go Tag^{R} Green Master Mix **(Promega).**

3- 1.5 µl of each primer solution AFU 7S, AFU 7AS (10 pmol/ml).

4- Remaining 6 µl volume was fulfilled by nuclease free water.

B) Second round PCR

The total reaction volume for the second – round PCR amplification is 20 ml which consist of:

1- 2 µl of first – round PCR product.

2- 10 µl of Go Tag^{R} Green Master Mix **(Promega).**

3- 1.5 µl of each primer solution AFU 5S, AFU 5AS (10 pmol/ml).

4- Remaining 6.5 µl volume was fulfilled by nuclease free water.

Amplification of AFU gene in thermal cycler:

The prepared PCR tubes with master mixture were placed in thermal cycler (Peco, Germany) which was programmed according to manufacturer's instruction.

A) first round PCR amplification

Initial denaturation at 94^{o}C for 2 minutes, then 26 cycles of denaturation at 94^{o}C for 40 sec., annealing at 65^{o}C for 1 min., extension at 72^{o}C for 1 min., and final extension at 72^{o}C for 5 minutes.

B) second round PCR amplification

Initial denaturation at 96^{o}C for 2 minutes, then 30 cycles of denaturation at 96^{o}C for 20 sec., annealing at 65^{o}C for 30 sec., extension at 72^{o}C for 30 sec., and final extension at 72^{o}C for 5 minutes.

Detection of Amplified DNA

By gel electrophoresis (1.5% agarose gel) **(thermo scientific, fermentas, UE)** and visualization under UV lights by transilluminator at302 nm by using GeneRuler^{TM} 100 pb plus DNA ladder **(thermoscientific, fermentas, UE)** Electrophoresis was carried out at 100 volts for 15 minutes then at 80 volts for 25 minutes. The gel was examined for bands of **249 bp**then photographed by a digital camera and data was transferred to computer for further documentation.

3. Results

Table (2): Demographic data and type of leukemia of the studied cases

	The studied cases N = 36	
Age (years)		
X ± SD	4.96±2.60	
Median	4.0	
Range	3 – 14	
	No	**%**
Sex		
Male	18	50
Female	18	50
Diagnosis		
ALL	29	80.6
AML	6	16.7
CML	1	2.8

X= Mean, **SD** = Standard deviation; **N** = Number

In table (2):-

- The age of the studied cases ranged from 3-14 years, and mean ± SD was 4.96 ± 2.60.
- The studied cases were 18 males (50%) and 18 females (50%).
- The studied cases were 29 ALL (80.6%), 6 AML (16.7%) and 1 case was CML (2.8%).

Table (3): Classification of studied patients according to EORTC / MSG definition of IFIS

	The studied cases N= 36	
	No	**%**
Possible IFD	24	66.6%
Probable IFD	10	27.8%
Proven IFD	2	5.5%

In table (3): There were 36 patients whose clinical, radiological or mycological signs indicated suspicion for IA. The studied patients, according to EORTC/MSC criteria, were classified as follows: 2 proven and 10 probable and 24 possible.

In table (4):-There is one case with positive air crescent on chest X-ray and dense well-circumscribed lesions with a halo sign on chest C.T scan, the same case showed aspergillus hyphae in BAL by

histochemical examination. There is also another case that showed sinusitis by C.T, the same patient had histopathological proof of aspergillosis.

Table (4): Imaging and histopathological results among the studied cases

	The studied cases N = 36	
	No	**%**
Chest X ray		
*Positive air crescent	1	2.8%
*Negative air crescent	35	97.2%
CT for chest		
***positive sign for aspergillus	1	2.8%
* negative sign for aspergillus	35	97.2%
CT for CNS		
*Free	36	100%
*Focal lesions	0	0%
CT forsinus		
*Sinusitis or Extension across bony barriers	1	2.8%
*Free	35	97.2%
Aspergillus hyphae in BAL		
***YES**	1	2.8%
***NO**	35	97.2%
Histological or cytopathologic proof		5.5%
***YES**	2	94.5%
***NO**	34	

N.B Positive sign for aspergillus, means (dense well-circumscribed lesions with or without a halo sign or air-crescent sign and cavity)

Table (5): Blood Culture results among the studied cases

	The studied cases N = 36	
	No	**%**
Culture		
Negative	8	22.2%
Candida	16	44.4%
Aspergillus	2	5.5%
bacteria	10	27.7%

In table (5):

Blood culture results for fungus showed 2 cases positive for aspergillus (5.5%),16 (44.4%) positive for candida, 10 (27.7 %) for bacteria and 8 (22.2%) were negative.

Table (6): Detection of aspergillosis by galactomannan antigen test among the studied cases.

	The studied cases N = 36	
	No	**%**
Galactomannan		
Positive	16	44.4
Negative	20	55.6
Galactomannan index		
X ± SD	1.12±1.36	
Median	0.25	
Range	0 – 3.9	

In table (6):-

There were 16 (44.4%) of the studied cases with positive galactomannan test and 20 (55.6%) with negative results by the same test.

The galactomannan index of the patients ranged from 0 – 3.9, and mean ± SD was 1.12 ± 1.36.

Table (7): Detection of aspergillosis by nested PCR among the studied cases.

	The studied cases N = 36	
	No	**%**
Nested PCR		
Positive	13	36.1
Negative	23	63.9

N.B: The lowest limit for nested-PCR was found to be 1cfu/ml.

In table (7): There were 13 (36.1%) of the studied cases with positive aspergillus by nested PCR and 23 (63.9%) were negative.

Table (8): Comparison between galactomannan and PCR results for detection of aspergillus among the studied cases

	Nested PCR					
	Positive N = 13		**Negative N = 23**		**Total**	
	No	**%**	**No**	**%**	**No**	**%**
Galactomannan						
Positive	13	100	3	13	16	44.4
Negative	0	0	20	87	20	55.6

In table (8): There were 3 cases which were positive for aspergillosis by GM test but negative by nested PCR.

Table (9): Nested PCR results as regards age, sex and diagnosis of the studied cases

	Nested PCR results				**Test**	**P value**
	Negative N = 23		**Aspergillus N = 13**			
Age					U	
X ± SD	4.96±3.13		4.96±1.3		1.39	0.16
Median	4		5			NS
Range	3 – 14		3 – 7			
	No	**%**	**No**	**%**	**X^2**	
Sex						
Male	11	47.8	7	53.8	0.12	0.73
Female	12	52.2	6	46.2		NS
Diagnosis						
ALL	20	87.0	9	69.2	3.32	0.19
AML	2	8.7	4	30.8		NS
CML	1	4.3	0	0		

X= mean, SD = Standard deviation, U = Mann Whitney U, X^2 = Chi square test, P value >0.05 = non significant

In table (9): There was non-significant difference between aspergillus infected patients and negatives by nested PCR regarding age, sex and type of leukemia **(P – value < 0.05).**

Table (10): Nested PCR results as regards temperature and number febrile attacks

	Nested PCR results				**Test**	**P value**
	Negative N = 23		**Aspergillus N = 13**			
Temperature						
X ± SD	38.99±0.50		39.59±0.29		U	<0.001
Median	39		39.5		3.57	HS
Range	38 – 39.7		39 – 40			
	No	**%**	**No**	**%**	**X^2**	
Number of febrile attacks						
1	1	4.3	0	0		
2	10	43.5	2	15.4	7.46	0.11
3	10	43.5	7	53.8		NS
4	1	4.2	4	30.8		
7	1	4.3	0	0		

X= mean, SD = Standard deviation, U = Mann Whitney U, X2 = Chi square test, P value >0.05 = non significant, HS = highly significant

In table (10): There was non-significant difference between negative and aspergillus positive nested PCR results as regards number of febrile attacks **(P-value = 0.11).**

While, there was highly significant difference in the mean value of temperature between positive and negative aspergillus patients by nested PCR **(P value <0.001).**

In table (11): Comparing the positive and negative aspergillus cases by nested PCR as regards WBC count and platelets it was statistically non-significant ***(P-vale > 0.05).*** While on comparing the same subgroups as regards ANC, it was statistically significant ***(P value = 0.03).*** There was highly statistic significant difference in the mean value of Hb between the two subgroups **(P-value <0.001).**

The ANC of aspergillus infected patients by nested PCR ranged from 250-490 cells/mm^3 and mean ± SD was 375.76 ± 90.18.

Table (11): Nested PCR results as regards CBC results

	Nested PCR results		U Test	P value
	Negative N = 23	**Aspergillus N = 13**		
ANC				
X ± SD	652.17±391.63	375.76±90.18	2.21	0.03
Median	490	380		S
Range	250 – 1400	250 – 490		
Hb				
X ± SD	7.78±1.70	6.0±0.75	3.78	<0.001
Median	7.5	6		HS
Range	4 – 11.5	4 – 7		
WBCs				
X ± SD	9404..35±5952.73	6542.31±5228.52	1.91	0.06
Median	7000	4500		NS
Range	2500 – 20000	2000 – 18000		
Platelets				
X ± SD	121.87±79.14	78.77±41.70	1.48	0.14
Median	106	90		NS
Range	20 – 300	15 – 150		

X= mean, SD = Standard deviation, U = Mann Whitney U, P value >0.05 = non significant, HS = highly significant
NC absolute neutrophilic count

Table (12): Nested PCR results as regards galactomannan index

	Nested PCR results		U test	P value
	Negative N = 23	**Aspergillus N = 13**		
Galactomanan				
X ± SD	0.23±0.45	2.68±0.93	4.85	<0.001
Median	0.1	2.5		
Range	0 – 2	0.9 – 3.9		

In table (12): Comparing the positive and negative aspergillus patients by nested PCR regarding to galactomannan index it was statistically significant **(P-value < 0.001).**

Table (13): Validity of galactomannan for diagnosis of aspergillus infection

	Galactomannan
AUC	0.985
P value	<0.001
Cut off point	0.80
Sensitivity	100%
Specificity	91.3%
PPV	86.7%
NPV	100%
Accuracy	94.4%

AUC = area under the curve.

Figure (3) and table (13) show galactomannan test validity for diagnosis of aspergillus infection with AUC = 0.985, and the best cut off level of serum galactomannan was 0.8, where sensitivity was 100% and specificity was 91.3%, PPV 86.7%, NPV 100%and accuracy of 94.4%.

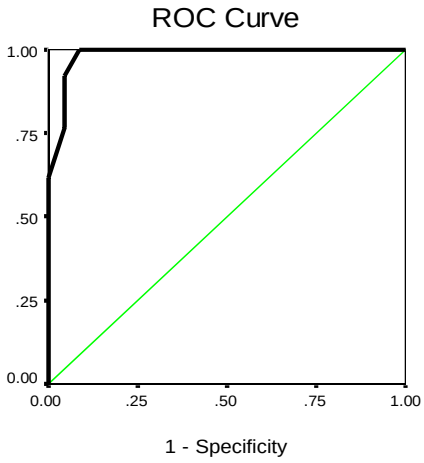

Figure (3): ROC curve of galactomannan test for diagnosis of aspergillus infection

Table (14): Validity of nested PCR for diagnosis of aspergillus infection

Item	TP	FN	TN	FP	Sens.	Spec.	PPV	NPV
Nested PCR	12	2	21	1	85.7%	95.5%	92.3%	%91.3%

In table (14): Show nested PCR validity for diagnosis of aspergillus infection where sensitivity was 85.7% and specificity was 95.5%, PPV 92.3%, NPV % 91.3%.

Figure (4): Agarose gel electrophoresis of the nested PCR

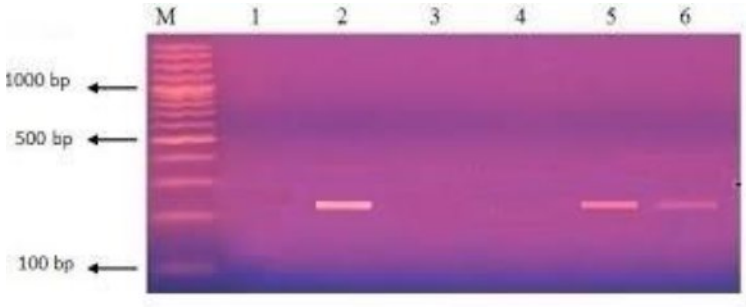

Figure (4): Agarose gel electrophoresis showing the nested PCR products: Lane M, GeneRuler TM 100 pb plus DNA ladder, Lanes 2,5,6 showing positive cases for *AFU* gene (249 bp), lane 3,4 showing negative control

4. Discussion

The diagnostic delay of invasive aspergillosis (IA) may be caused by that the typical clinical signs according to EORTC/MSG criteria are non-visible and often develop too late in the course of infection ***(Brook et al., 2009).***

The diagnosis of invasive aspergillosis can be established by the presence of *aspergillus* in sputum, blood and BAL by microscopy or culture. However, these methods are time consuming and misidentification can usually occur because some fungi may be poorly characterized by inexperienced persons ***(Arvanitis et al., 2015).*** Although, tissue biopsy and fluids obtained by invasive procedures (transthoracic percutaneous needle aspiration, video assisted thoracoscopic biopsy, bronchoscopy) are considered the cornerstone of diagnosis, they are not always possible to obtain from patients who are not fit (hypoxemic, hemodynamically instable an thrombocytopenic*)* ***(De Pauw et al., 2008)*** and ***(Hummel et al., 2009).***

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 ***(Lopes da Silva et al., 2010).***

Chest radiography is too insensitive to diagnose IA at an early stage and chest CT scan, although an important diagnostic tool, it remains unspecific since initial nodular lesions surrounded by lower attenuation "halo sign" and late cavitations following neutrophil recovery creating the "air crescent sign", may appear due to other species of fungi (*Zygomycetes, Fusarium, Scedosporium*) and even bacteria (*Pseudomonas aeruginosa*), and in several occasions these features are not present at all ***(Brook et al.,2009).***

Furthermore, blood cultures are not useful since they are often negative even in disseminated infection and take too long to become positive (median 15 days) ***Lopes da Silva et al., 2010).***

It is clearly now that it is urgent 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.

The aim of this work is to detect the incidence of invasive aspergillosis (IA) among children with hematological malignancies, and to compare the diagnostic performances of galactomannan antigen test by ELISA kit, and detection of aspergillus DNA by nested-PCR in order to choose early and rapid method for diagnosis for IA in a trial to improve prognosis and reduce the use of antifungal drugs.

In the present study candida was more commonly isolated 16 (44.4%) by blood culture for fungus than aspergillus 2 (5.5%). Nearly same results were obtained by ***Shi et al., (2015)*** who reported that: candida is the most common pathogen for early invasive fungal infection (IFI), and also by ***Sahbudak et al., (2015)*** who concluded that the most isolated

agents among leukemic children with IFI were candida spp.

This also was in agreement with ***Badiee et al., (2012),*** who found candida in 72.2% andaspergillus in 27.3% of their samples.

On the other hand, ***Walsh and Groll, (1999) and Direkel et al., (2012)*** reported that molds are widely distributed in nature and aspergillus spp. represent the most frequently observed causative agent of IFIs in patients with hematological malignancies and the most common cause of mortality in children with leukemia, while candida spp., constitute the second most common cause of blood stream infection.

Some studies do not rely upon the results of blood culture at all as ***El-Sayed et al., (2012)*** study who found that detection of fungemia by means of fungal blood culture is notoriously difficult and IFIs are diagnosed better by molecular assay as naked DNA can be detected by PCR due to the presence of dead and degrading fungi within circulating phagocytes, and ***Gurtner et al., 2007*** who found that blood samples showed no growth of aspergillus which was confirmed only by PCR and histology.

In our study, the two positive blood culture for aspergillus are considered contamination and canceled, as recovery of *aspergillus* species from blood cultures of proven or probable cases are invariably represents contamination according to EORTC / MSG definition of IFIS ***(Ascioglu et al, 2002).***

Aspergillus galactomannan test (GM) is a serological test (ELISA) aids in the diagnosis of invasive aspergillosis and assessing response to therapy. Galactomannan is a heat stable and water soluble cell wall polysaccharide that is released by *Aspergillus* species during fungal growth.

In the present study we used aspergillus galactomannan antigen test for detection of invasive aspergillosis (IA). GM cut-off value is an important factor in determining the sensitivity and specificity of the test. By using cut-off 0.8; 16 (44.4%) of cases had positive results and 20 (55.6%) of cases had negative results.

The ROC curve revealed that the galactomannan Ag test for serum revealed 100% sensitivity and 91.3% specificity at optimal GMI cut-off of 0.8. A decade ago, a cut off serum ratio of 1.5 was recommended in the manufacturer's manual in Europe. However, currently many studies have used a cut off value of 1, and the US food and Drug Administration in the United States recommended a cut-off value of 0.5. The GM cut-off of 0.8 used in our laboratory increases the sensitivity at the cost of lowering the specificity but was agreed as the ideal threshold by the EORTC/MSG criteria. Actually, weal so faced improved accuracy to 94.4% of the test with higher threshold, when we use cut-off of 0.8. In addition, in this study the negative predictive value of GM was 100%, which means that GM test is very good test to rule out IFI.

Our findings are in agreement with ***Sarrafzadeh et al., (2010)*** and ***Pfeiffer et al., (2006)*** who found serum GM test had a sensitivity of 99% and specificity of 90% for cases of invasive aspergillosis.

Also, these results were similar to that reported by ***White et al., (2015),*** they founded that GM sensitivities and specificities for diagnosing IA were 98% and 91% and had reported that GM and PCR is now mature enough for inclusion in the EORTC / MSG definition.

In contrast to our study ***El-Mahallawy et al., (2006)*** reported that neither the sensitivity nor specificity of GM test was sufficient ***Scotter et al., (2005)*** also reported that the GM assay exhibited poor sensitivity but high specificity.

This variation in the results of GM results can be due to variation in the study population and types of samples.

Several factors limiting the specificity of the GM EIA in this study and other studies as false-positivity, which can be caused by treatment with some antibiotics of fungal origin such as amoxicillin-clavulanate, piperacillin-tazobactam, and beta-lactams, even up to five days after the cessation of treatment ***(Badiee et al., 2012),*** also immunoreactivity with other organisms, such Penicillium spp., Paecilomyces spp., Blastomycesdermatitidis, Nigrospora and Trichothecium, false-positive results can be also caused by contamination by GM found in dietary products as in pasta, cereals and formula milk ***(Dornbusch, et al., 2010)***, or translocation of GM across the intestinal wall if chemotherapy-induced mucositis is present ***(Hummel et al., 2009).***

PCR-based techniques that target DNA have a high degrees of sensitivity and specificity and provide a method for early diagnosis of invasive aspergillosis ***(Arvanitis et al., 2015).***

In the present study we used nested PCR for detection of IA among the studied cases, 13 (36.1%) of the cases were positive for aspergillus and 63.9% were negative.

This study showed that there were no significant differences of the age of the patients, their sex and type of leukemia in patients with IA proved by nested PCR when compared with negative patients (p value > 0.05), these results were in agreement with ***Snydman, (2012)*** and ***El-Sayed et al., (2012),*** who reported that age, sex and type of leukemia are not accurate predictors of IFIs in children with leukemia.

Febril neutropenia (FN) is a clinical syndrome as defined by Infectious Diseases Society of America (IDSA), refers to fever in patients who have an

absolute neutrophil count < 0.5 × 10^9/L that is predicted to fall below < 0.5 × 10^9/L within 48 hours of onset of fever ***(Keng and Sekeres, 2013).***

FN following chemotherapy in patients with malignant haematological diseases is classified in three categories, microbiologically documented infections, subdivided into those with and without blood stream infection, clinically documented infection, and fever of unknown origin. ***(Demirel et al., 2015)***.

In this study, the temperatures of patients were highly significantly higher in patients with IA proved by nested PCR when compared with negative patients (p value < 0.001) and the count of neutrophil was significantly lower in PCR proven IA when compared with negative patients (p value < 0.05). These results were in accordance with ***Kobayashi et al., (2008),*** who concluded that temperature increases in all cases of IA and neutrophil counts decrease in all cases of IA. They also reported that the most common presentation in PCR proven IA in immunodeficient patients were fever and severe degree of neutropenia, and also in agreement with ***Lucas et al., (1996)*** and ***Portugal et al., (2009)*** who found an association between fungal infections and decreased count of neutrophilless than 100after 48 hours.

The children with leukemia in the study of ***(Dotis et al., 2007),*** have the major risk factor for developing CNS aspergillosis which was severe neutropenia secondary to chemotherapy. Neutropenia and corticosteroids may synergistically act to depress natural host defenses.

In contrast to this, the study ***of El-Sayed et al., (2012)*** who concluded that fever persisting at day 4 of admission, together with absolute monocytic count < 100/mm3 increased the risk for IFI in children with cancer. Indeed there was no significant difference in absolute neutrophil count between patients with and without fungal infection, in the same time he has been previously found that, in AML patients, the intensity of neutroperia can be a good predictor for IFI.

The obtained results showed that Hb was significantly lower in patients with positive fungal infection proved by nested PCR when compared with negative patients, ***Sun et al., (2009***) and ***Mitrovic et al., (2012),*** supported these results.

Grigull et al., (2003) results were in accordance with results of present study, and they stated that there was no significant difference between control group and patients with invasive fungal infection as regards platelets count, in the same time, his study was different from the present study in that there was no significant difference between the 2 group as regards Hb level.

The obtained results showed that galactomannan index was significantly higher in patients with aspergillosis proved by nested PCR when compared with negative patients (p value < 0.001). ***Han et al., (2015)***, Supported these results in that high galactomannan index is found in patients with IA when compared with control group. They found that high serum galactomannan index was associated with mortality in immunocopromised children due to IA. In the same time. There were 3 cases which were positive for aspergillosis by GM test but negative by nested PCR, this can be caused by false positivity of GM test as mentioned before.

Direct comparisons between molecular methods and the GM assay for the detection of aspergillosis do not yield consistent results as to which test is best. This can be to that, GM and other fungal antigens were released when Aspergillus was found in exponential growth phase, while fungal DNA was released when the hyphae break up, a phenomenon which occurs naturally by autolysis when the amount of nutrients is limited or when antifungal agents are present (***Mennink-Kersten et al., 2006***).

In this study nested PCR showed sensitivity, specificity, PPV, NPV of 85.7%, 95.5%, 92.3%, 91.3% respectively. This is similar to the study of ***Lopes da Silva, 2010*** where the serum PCR assay had a sensitivity of 75.0% and a specificity of 91.9% and ***Badiee et al., 2012*** where the sensitivity, specificity, negative and positive predictive values were, 80%, 96.2%, 88.9%, 92.6%, respectively.

We can clearly see low sensitivity of nested PCR, this can be explained by increased false negativity (as there were two patients with positive IA according to EORTC/MSG criteria but negative PCR results) which may be due to that there may be no DNA aemia at the time of blood sampling, so that aspergillus DNA was not present in blood samples or the level of DNA aemia was below the detection threshold of the PCR assay. There is still relatively poor understanding of DNA release and kinetics in fungal infections ***(Hummel et al., 2009).*** False negative results of polymerase chain reaction may also be caused by antifungal therapy, which can be associated with conversion of PCR tests to negative ***(Buchheidt et al, 2004).***

Low sensitivity of PCR supports the hypothesis that investigating samples from the site of aspergillus infection is moresensitive than blood sampling ***(Hummel & Buchheidt, 2007).***

On other hand, ***Suarez et al.*** report that in comparison to GM test, the PCR assay was found to be highly sensitive and specific for the early diagnosis of IA in high-risk patients with hematological disorders ***(Suarez et al., 2008).***

Although the specificity of PCR assay for diagnosis of patients for IA is higher than specificity of GM test, but it is still lower than expected it may be

reduced by potential 'false-positive' results (as one patient without clinical signs of IA was positive with nested PCR), which can caused by transient aspergillus DNA emia of sub-clinical infection during the time of the test then this transient DNA emia disappear by empirical antifungal therapy or neutrophil recovery, or hepatic clearance of fungal elements from the blood.

Environmental contamination by fungal spores is another possible cause of 'false-positive' results ***(McClintock et al, 2004)*** and ***(Halliday et al., 2005***). This was not likely to have occurred in the present study as strict precautions were taken, including using laminar airflow, processing each specimen with negative control and a positive control of fumigates DNA during DNA extraction and PCR amplification.

Small sample volumes, is an another benefit of using PCR as low as 0.5 ml were sufficient for DNA detection. This is of advantage in small children where it is difficult to obtain appropriate specimens due to their small size ***(Roilides, 2006).***

In addition, nested PCR results can be obtained earlier than other tests. According to ***Estrella et al., 2009***, fungal DNA was detected by PCR much earlier than GM in patients with aspergillosis and than HRCT and preceded standard diagnosis of IA by about of 10-14 days.

Although, the cultures may be positive in proven and probable cases (sensitivity and specificity were 100%), but this method cannot help the diagnosis in possible patients for whom clinical sampling such as bronchoalveolar lavage or biopsy is not available, because it is invasive and not feasible in neutropenic patients. ***Badiee et al. 2012***. Ideally the same patient should be tested by PCR and GM at the same time. This diagnostic approach lead to progressive success in solving this serious complication in adult and children ***(White et al., 2015)***.

Conclusion:

1-Our data revealed that the incidence of invasive aspergillosis is high among children with hematological malignancies about (36%) and it is a serious problem.

2-GM test alone has limitations for the diagnosis of IA, due to its false positive results and decreased specificity.

3-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 antifungal therapy in addition to clinical and radiologic findings.

4-Both tests enable us to follow up the disease by monitoring the efficacy of the therapy chosen and change to alternative antifungal drugs in case of no response.

5-There is a necessity for combination between these two diagnostic methods for early, rapid and accurate diagnosis of IA.

6-To increase the sensitivity of both PCR and GM testing, obtaining samples before starting of antifungal treatment and serial sampling are desirable.

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