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Research Article

Phenotypic and Molecular Detection of Invasive Pulmonary Aspergillosis from Inpatients Children with Hematologic Cancers

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Abstract

Objective: Invasive pulmonary aspergillosis (IPA) is a growing concern in immunocompromised children. Data from Middle East regarding burden of children's *Aspergillus* infections are scant. This study aimed to update the frequency and antifungal susceptibility profile of *Aspergillus* isolates obtained from children with malignant underlying diseases.

Materials/Patients/Methods: A total of 75 episodes from 60 patients with clinical presentation suggestive of IPA were recruited between January 2013 to December 2016, and clinical data were retrospectively collected. *In vitro* susceptibility was determined by E test method using Clinical and Laboratory Standards Institute guidelines.

Result: According to EORTC/MSG criteria, a total of 75 episodes, 16 (21.3%) and 59 (78.7%) cases with probable and possible IPA were identified, respectively. *Aspergillus flavus* was isolated in 7neutropenic patients showing and showed reduced susceptibility to amphotericin B. However, all isolates demonstrated susceptible profile to caspofungin (≤ 0.25 µg/ml), posaconazole (≤ 0.5 µg/ml) and voriconazole (≤ 2 µg/ml).

Conclusion: The *Aspergillus* species causing IPA in children with malignancies differed from those in Western countries. Our results provide useful information on local epidemiology of IPA and selection of proper empirical antifungal agent.

Keywords: *Invasive Pulmonary Aspergillosis; Children, Aspergillus flavus; Neutropenia; Iran*

Introduction

Invasive Aspergillusis (IA) is one of the most common and serious infectious of increasing importance in immunocompromised children, worldwide [1].

Early diagnosis is important as it leads to appropriate therapy and improves patient outcomes. Several diagnostic tests are available with varying degrees of sensitivity and specificity [2-4]. Challenges in definitive diagnosis arise due to nonspecific clinical manifestations, radiologic signs and practical difficulties in histopathological examination, (tissue biopsy and culture) [2,3,5]. Detection of galactomannan (GM) is one of the most useful tests and a recommended marker for the diagnosis of IPA in patients with impaired immune status, including those with different types of malignancies [6]. GM is a polysaccharide released during *Aspergillus* growth in different body fluids and can be detected in serum and broncho-alveolar lavage, (BAL). Double-sandwich ELISA has high sensitivity for detection of GM and has been approved by the FDA (Food and Drug Administration) during the last decades [2,5,7,8]. Different *Aspergillus* species vary in pathogenicity and antifungal susceptibility such as *A. tereus* and A*. nidulans;* these species are less susceptible to amphotericin B but *A. fumigatus* is different and more susceptible than the above mentioned species [9,10]. Molecular identification tests are performed by several methods such as PCR sequencing, PCR-Enzyme Immune assay (EIA), nested PCR, PCR-RFLP, real time and other molecular tests [9,11-13]. In addition, Clinical Laboratory Standard Institute (CLSI) has also developed standard and reproducible approach to evaluate antifungal susceptibility of the molds [14-16]. Of note, despite advances in the diagnosis and therapy of IA in the last 15 years, treatment success and long-term survival after IA diagnosis remain suboptimal [1].

Recently, Hedayati., *et al.* reported the annual incidence of IA in Adults with hematologic malignancy, lung cancer and chronic pulmonary obstructive disease in Iran [17]. However, data on pediatrics populations was lacking. In the present study, we therefore determined the clinical characteristics and antifungal susceptibilities of *Aspergillus* species in disseminated pulmonary infections of children with malignant disease in and to identify the potential risk factors associated with mortality and therapeutic failure.

Materials and Methods

Patients and method

From 2013 to 2016, 75 episodes admitted in Pediatric oncology department of Mofid children's hospital were evaluated for IPA. The manuscript 's ethic code is SBMU.REC.1392.65 and all the samples were gathering from inpatients Hematologic cancers who suspected to have fungal infections. Patients with clinical presentation suggestive of IPA such as cough, fever, dyspnea, chest pain and hemoptysis were followed and evaluated for IPA. CNS involvement or disseminated fungal infections were followed as well. We filled in a questionnaire about risk factors such as chemotherapy, prolonged neutropenia (< 500/µl) for more than 10 consecutive days, persistent fever (more than 96hr.) not responding to broad-spectrum antibacterial therapy, and other criteria summarized on table 2 [18]. Clinical samples such as CSF, Blood, and sputum were sent to Pediatric Infections Research Center where this investigation was performed.

Direct microscopy of samples was performed and the specimens were cultured on Malt Extract Agar (MEA), incubated for 7 days at 30°C. GM detection was performed according to instruction of the (Platelia *Aspergillus* EIA Bio-Rad) kit, Cutoff ≥ 0.5 was considered as a positive GM test. Patients were classified according to EORTC/MSG criteria as possible and probable of infection (Table 1) [3,6]. Positive Cultures were identified by phenotypic (macroscopic and microscopic) and molecular (PCR- RFLP) criteria and antifungal susceptibility test was done.

	EORTC criteria
Proven	Existence of Hyphae and tissue damage in histopathology or cytopathology damage or positive culture of that specimen with abnormal radiologic[#] or clinical criteria of infection
Probable	Host factor* with one positive mycological examination (direct smear, Culture, antigen assay[$]) and clinical criteria of infection*
Possible	Host factor* with clinical criteria

Table 1: *Diagnostic EORTC criteria for Invasive pulmonary aspergillosis [3,6].*

[#]Abnormal radiologic criteria.

**Host factor: Prolonged neutropenia (< 500 /mm^3 for 10 days), allogeneic stem cell transplantation (SCT), prolonged use of corticosteroids (0.3 mg/Kg/day > 3 weeks), use of immunosuppressants during the past 90 days and inherited immunodeficiency [34].*

[$]Positive antigen assay: Galactomannan or β-D glucan.

Molecular identification

Aspergillus species were grown on MEA for 3 - 7 days. DNA extraction was performed by using glass-bead and grinder for disruption and phenol chloroform method. Finally, dried DNA was suspended in 50 µlit 0f double distilled water and stored at -20°C for future use [11,19]. We used ITS1 5´-TCCGTAGCTGAACCTGCGG-3´ and ITS4 5´-TCCTCCGCTTATTGATATG-3´ primers and *HhaI* enzyme [19]. ITS region was amplified in final volume of 100 µl (1 µl DNA template, 0.20 µM each forward and revers primers, each dNTP 0.10 mM, 2.5U Taq DNA polymerase). PCR amplification was performed by 30 cycles as following steps: initial denaturation (94°C-5 minutes), annealing (54°C-45s), extension (72°C-1 minute) and final extension (72°C-7minutes). PCR products were visualized in gel electrophoresis (1%) with TBE buffer and 0.5 µg ethidium bromide. Obtained PCR products were digested by *HhaI* enzyme (0.5 µl *HhaI* enzyme, 1.5 µl Tango buffer and 8 µl Molecular grade water) then incubated at 37°C for 2h. DNA fragments were separated and visualized at 2% agarose gel after 2 hours 100V. We specified obtained PCR products and fragments after PCR- RFLP process according to the molecular size of the markers. The species were identified according to banding pattern of the PCR products and fragments after digestion by *HhaI* enzyme [19].

Antifungal susceptibility E test

E-test was performed according to manufactures' instruction (AB BIODISK Solna, Sweden) to assess *in-vitro* antifungal susceptibility of the strains. The isolates were cultured on potato dextrose agar at 35°C for three to seven days (good sporulation at 48 hours) to stimulate sporulation. Media for E tests contains of RPMI 1640 broth (sigma chemical co.) with L-glutamine without bicarbonate, morpholine-propane-sulfonic acid MOPS (Sigma, St. Louis, MO.), 1.5% bacto agar and 2% glucose with PH = 7. Suspension was prepared by scraping surface of colonies by loop then mixed with sterile saline solution with tween80. After settling the suspension, supernatant was transferred to sterile tubes and inoculum suspension adjusted at approximately 10^6 CFU/ml. The prepared suspensions were inoculated on plates by sterile swabs in three directions. We let the plates to dry in approximately 15 minutes, placed E test strips on them and then incubated these at 35°C for 24-48h. Complete (100%) inhibition for Amphotericin B and (80%) of growth for Azoles was considered as MIC [14,15]. We reported minimum effective concentration (MEC) as end point for caspofungin and micro colony in inhibition area was ignored [20]. We used *C. parapsilosis* (ATCC 22019) as quality control parallel with series of tests (Figure 1).

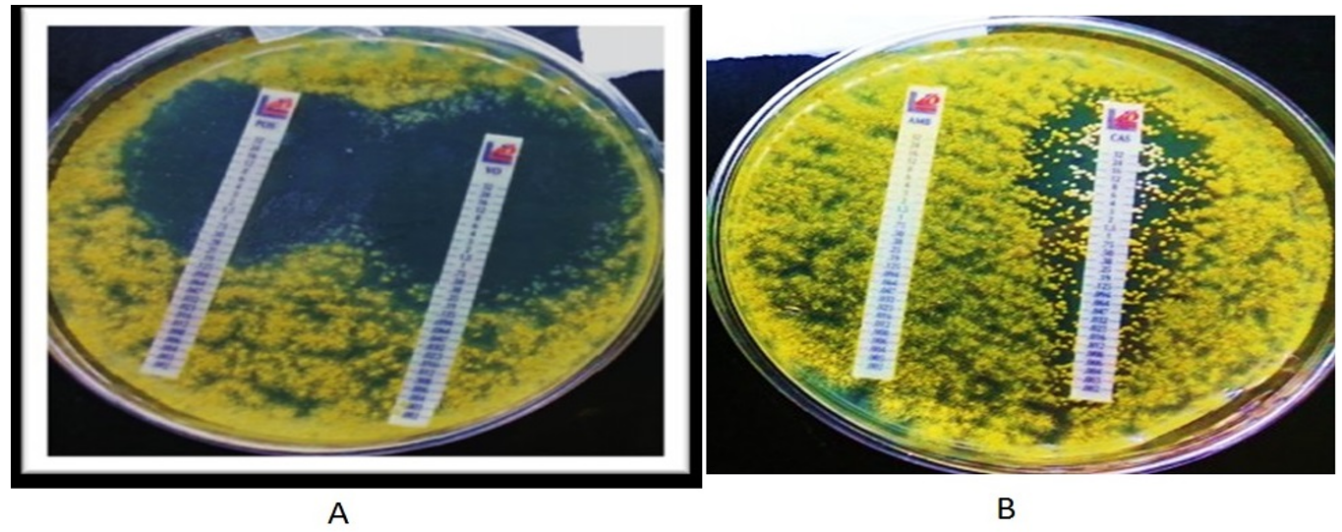

Figure 1: *A: E test growth inhibition for posaconazole and voriconazole, B: E test growth inhibition for amphotericin B and Caspofungin. Minimum Effective Concentration (MEC).*

Results

During the study period, 75 episodes of IPA caused by *Aspergillus* spp. were identified. From a total of 75 episodes, 16 (21.3%) and 59 (78.7%) were classified according to EORTC/MSG criteria as probable and possible, respectively [3]. However, it was not possible to classify proven cases of IPA due to lack of biopsy samples. The clinical characteristics and signs and symptoms of the patients are summarized in table 2. The most common host factors were neutropenia 46 patients, (59.7%) and receiving chemotherapy 49 patients, (81.7%). Neutropenia was seen in 12 (75%) and 35 patients (55.7%) in the probable and possible groups, respectively. Thirteen patients (92.9%) in the probable group and 36 (76.6%) in the possible group were receiving chemotherapy; 13 of probable cases (81.3%) were GM positive and 7 were culture positive (43.8%). Chest X ray abnormalities and CT-scan changes are summarized in table 3 and 4. Out of 75 cultured specimens, seven were positive, identified as *A. flavus* by macroscopic, microscopic and PCR-RFLP test.

	Invasive pulmonary aspergillosis		
	Probable 16 (21.3%)	**Possible 59 (78.7%)**	**Total 75 (100%)**
Age (month)	70.32 ± 50.76	66.57 ± 56.09	67.44 ± 54.50
Gender			
Female	9 (64.3%)	21 (45.7%)	30 (50%)
Male	5 (35.7%)	25 (54.3%)	30 (50%)
Underlying disease			
AML	2 (14.3%)	11 (23.9%)	13 (21.7%)
ALL	8 (57.1%)	12 (26.1%)	20 (33.3%)
Wilms Tumor	0 (0%)	7 (15.2%)	7 (11.7%)
NHL	2 (14.3%)	2 (4.4%)	4 (6.7%)
Rhabdomyosarcoma	0 (0%)	2 (4.4%)	2 (3.4%)
Ewing's sarcoma	0 (0%)	1 (2.2%)	1 (1.7%)
Germ cell tumor	1 (7.1%)	3 (6.5%)	4 (6.7%)
Primitive neuroectodermal tumor	0 (0%)	1 (2.2%)	1 (1.7%)
Langerhans cell Histiocytosis	0 (0%)	1 (2.2%)	1 (1.7%)
Peritoneum carcinoma	0 (0%)	1 (2.2%)	1 (1.7%)
Neuroblastoma	1 (7.1%)	5 (10.9%)	6 (10%)
Risk factors			
Neutropenia	12 (75%)	34 (55.7%)	46 (59.7%)
Use of corticosteroid	4 (28.6%)	10 (21.7%)	14 (23.3%)
Chemotherapy	13 (92.9%)	36 (76.6%)	49 (81.7%)
Clinical signs			
Cough	3 (18.8%)	8 (13.1%)	11 (14.3%)
Fever	12 (75%)	37 (60.7%)	49 (63.6%)
dyspnea	0 (0%)	0 (0%)	0 (0%)
Tachypnea	0 (0%)	3 (4.9%)	3 (3.9%)
Chest pain	0 (0%)	1 (1.6%)	1 (1.3%)
Respiratory rate	28.33 ± 16.46	28 ± 10.19	28.7 ± 11.45
GM	13 (81.3%)	0 (0%)	13 (17.3%)
Culture	7 (43.8%)	0 (0%)	7 (9.1%)
Chest- X- ray abnormality	8 (50.0%)	39 (63.0%)	47 (61.0%)
Chest CT- scan abnormality	6 (37.5%)	30 (49.2%)	36 (46.8%)
Antibiotic prophylaxis	2 (12.5%)	15 (24.6%)	17 (22.1%)
Antifungal prophylaxis			
Fluconazole	1 (6.3%)	5 (8.2%)	6 (7.8%)
Voriconazole	0 (0%)	8 (13.1%)	8 (10.4%)
Posaconazole	1 (6.3%)	0 (0%)	1 (1.3%)
Antifungal therapy			
Amphotericin B	1 (6.3%)	1 (1.6%)	2 (2.6%)
Caspofungin	0 (0%)	1 (1.6%)	1 (1.3%)
Voriconazole	2 (12.5%)	7 (11.5%)	9 (11.7%)
Posaconazole	0 (0%)	0 (0%)	0 (0%)
Death	6 (37.5%)	17 (27.9%)	23 (29.9%)

Table 2: *Characteristic, clinical, radiologic and microbiologic findings of 75 episodes.*

CX Ray	Probable N (%)	Possible N (%)	Total N (%)
Infiltration	1 (6.3%)	17 (27.9%)	18 (23%)
Consolidation	0 (0%)	7 (11.5%)	7 (9.1%)
Nodular finding	1 (6.3%)	3 (4.9%)	4 (5.2%)
Lymphadenopathy	0 (0%)	0 (0%)	0 (0%)
Pneumothorax	0 (0%)	4 (6.6%)	4 (5.2%)
Pleural effusion	3 (18.8%)	7 (11.5%)	10 (13%)
opacities	1 (6.3%)	14 (23%)	15 (19.5%)
other	4 (25.0%)	9 (14.8%)	13 (16.9%)

Table 3: *Chest-X-ray findings 61 patients.*

	Probable N (%)	Possible N (%)	Total N (%)
Pleural Effusion	0 (0%)	2 (3.3%)	2 (2.6%)
Ground Glass	3 (18.8%)	6 (9.8%)	9 (11.7%)
Nodule	4 (25.0%)	15 (24.6%)	19 (24.7%)
Opacity	2 (12.5%)	5 (8.2%)	7 (9.1%)
Hemorrhage	0 (0%)	1 (1.6%)	1 (1.3%)
Cavitation	1 (6.3%)	3 (4.9%)	4 (5.2%)
Halo Sign	0 (0%)	0 (0%)	0 (0%)
Infiltration	0 (0%)	2 (3.3%)	2 (2.6%)
Consolidation	1 (6.3%)	2 (3.3%)	3 (3.9%)
Other	2 (12.5%)	9 (14.8%)	11 (14.3%)

Table 4: *Chest CT-scan findings 61 patients.*

The results of *in vitro* antifungal susceptibility for 7 *A. flavus* species are presented in table 5. Amphotericin B demonstrated the widest range of MIC (1 - 4 µg/ml). However, all isolates had low MIC of voriconazole (≤ 2 µg/ml) and posaconazole (≤ 0.5 µg/ml) MIC results for Caspofungin were ≤ 0.25 µg/ml. According to obtained results Caspofungin was the most active antifungal agent followed by posaconazole, voriconazole and amphotericin B.

Isolates (n)	Antifungal agents	MIC range (µg/ml)	MIC50	GM[b]	% ≤ ECVc
A. flavus (7)	Amphotericin B	1 - 4	2	2.24	100
	Voriconazole	0.094 - 0.38	0.25	0.25	100
	Posaconazole	0.047 - 0.38	0.094	0.09	100
	Caspofungin[a]	0.032 - 0.25	0.064	0.08	100

Table 5: *In vitro antifungal susceptibility of A. flavus isolates: Minimum Inhibitory Concentration (MIC) range, MIC50, Geometric Mean (GM).*

[a]*: For caspofungin MEC (minimum effective concentration).*

[b]*: Percentage of MICs less than or equal to ECV (ECV = 4 µg/ml for amphotericin, 2 µg/ml for voriconazole, 0.5 µg/ml for posaconazole and caspofungin.*

As shown in table 2, neutropenia was the main underlying diseases in children with positive A. fumigatus identification.

Discussion

IPA is a serious the main cause of IA in high risk patients with hematologic malignancies [21]. IA is a life threatening opportunistic fungal infection and despite advances in therapy has high mortality and morbidity [2,8,22]. Early diagnosis and identification of IPA is very important. In our investigation in 75 samples we found 16 (21.3%) probable and 59 (78.7%) possible cases according to EORTC criteria. Few studies have been performed about IA in pediatric patients; 7.1% of 675 acute leukemia patients have been reported as probable or possible cases of IA, these figures are different from our investigation [23]. This difference may be because of the differences in the number and type of patients. In Badiee*., et al.* that was performed on 62 patients with hematologic malignancies in Shiraz that 11 cases (17.7%) with invasive *aspergillosis* were found including: 1 proven, 8 probable and 2 possible cases were found that was incompatible with our findings. These differences may be due to differences in diagnostic methods with various sensitivity and specificity [4].

Radiology is a noninvasive approach for diagnosis of IA. In our study infiltration and nodules were seen on chest x-ray in probable and possible cases but there were no air crescent and Halo signs as has been mentioned in references [6,24].

We detected serum galactomannan (GM) which is another non-invasive and useful diagnostic test and may be used as a prompt and promising complementary test besides other diagnostic approaches in immune deficient patients [25]. In the study by Sarrafzadeh., *et al.* [10] GM detection in serum had 71% sensitivity and 89% specificity, while our figures were 57% sensitivity and 86% specificity. Klont., *et al.* [26] mentioned a sensitivity between 50 - 92% and specificity of 94 - 99.6% that is higher than our results. Differences in obtained results are because of study group and number of samples. According to our data negative predictive value (NPV) and positive predictive value (PPV) for GM test were 95% and 30% respectively that were compatible with Sarrafzadeh., *et al.* results (92 - 98% NPV), (25 - 62% PPV) [8]. The advantage of GM detection in serum is to accelerate diagnosis before other presentation of fungal infection are evident [26].

According to our results *A. flavus* is the main cause of IPA in our patients. Studies have shown abundance of species isolated from clinical samples depends on the geographical area. *A. terreus* and *A. niger* were isolated as second most common species after *A. fumigatus* in a hospital of Austria and Spain respectively. Our study is an agreement with previous reports from Iran, highlighting that non-fumigatus *Aspergillus* species are the leading cause of *Aspergillus* infections in the tropical and subtropical Middle East. In a prospective study between 2015 and 2016, a total of 150 bronchoalveolar specimens was collected from patients suspected to pulmonary aspergillosis underlying immunodeficiencies in Mashhad, Northeastern Iran. Overall, *A. flavus* was the predominant cause of probable invasive pulmonary aspergillosis, followed by *A. tubingensis* and *A. fumigatus* [27]. Of note, *Aspergillus* species demonstrated various degree of susceptibility commercially available antifungals, therefore performing antifungal susceptibility tests is essential for treatment and surveillance programs. The epidemiologic cut-off values (ECVs) of *Aspergillus* isolates were determined for antifungals in 2016 by Espinel-ingroff., *et al.* ECVs for voriconazole was 2 µg/ml, posaconazole and caspofungin 0.5 µg/ml and amphotericin B 4 µg/ml [28].

In this study MIC was lower than ECVs reported for *A. flavus* species for voriconazole, posaconazole, Caspofungin and amphotericin B [28]. According to current study potent activity was observed or posaconazole and Caspofungin by MIC50 (0.094 µg/ml and 0.064 µg/ml) respectively followed by voriconazole and amphotericin B MIC50 (0.25, 2) to *A. flavus* species.

Similarly, Taghizadeh-Armaki., *et al.* studied the genetic diversity and *in vitro* antifungal susceptibility of 200 *A. flavus* strains originating from clinical and environmental sources, which were collected between 2008 and 2015; posaconazole and anidulafungin showed the

greatest *in vitro* activity among systemic azoles and echinocandins, respectively. However, the majority of the *A. flavus* isolates showed reduced susceptibility to amphotericin B [29].

Among the first (fluconazole, itraconazole) and second (voriconazole, posaconazole) group of Triazoles fluconazole is not effective on molds but itraconazole has wider range and is effective on yeast and *Aspergillus species* [30]. Itraconazole and voriconazole are used as first line antifungal agents for treatment of invasive aspergillosis caused by *A. flavus and A. fumigatus and* had good therapeutic results [30,31]. In the current study voriconazole and posaconazole have shown potent activity to *A. flavus* similar to results in other studies in Iran [15,31]. Amphotericin B is a polyene antifungal with fungicidal effect by broad spectrum activity and is used as the first antifungal for clinical purpose [30,31]. We obtained the widest range [1 - 4 µg/ml) and highest MIC (4 µg/ml) antifungal that is similar to Khodavaisy., *et al.* and Kachuei., *et al*. In Alborzi., *et al*'s study 36 *A. flavus* isolates from 66 were resistant to Amphotericin B [15,31,32]. Gonçalves., *et al.* reported limited potent activity (two fold higher than *A. fumigatus*) of Amphotericin B to *A. flavi* section [33]. Caspofungin is an echinocandin antifungal with fungistatic and broad spectrum activity. In this study caspofungin had effective activity on *A. flavus* isolates that is compatible with other studies in Iran [15,31,32]. In our investigation *A. flavus* species was susceptible to caspofungin, posaconazole, voriconazole and Amphotericin B; these agents may be considered by the clinicians according to their respective MICs to choose appropriate treatment.

Conclusion

In conclusion, Invasive aspergillosis is considered as one of the most important infections in patient under chemotropic and special with neutropenia [4]. The antifungal susceptibility of *A. flavus* isolates was not linked with the source of isolation or underlying disease of patients. The *Aspergillus* species causing IPA in Iranian children with malignancies differed from those in Western countries, which provide useful information about local epidemiology and selection of proper empirical antifungal agent.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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