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Diagnostic Aspergillosis In The Sputum Of Patients With Pulmonary Complains And Antifungals Susceptibility Of Triazoles And Caspofungin Using E-Test

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Abstract: Aspergillus infections are among the most feared opportunistic infections in humans because they are capable of causing several distinct pulmonary diseases .The aim of this study detection the aspergillosis occurrences with patients undergo pulmonary infection and Susceptibility testing of some antifungals by MIC and E-test . The results showed that 139 (44.6%) out of 311 patients were presented with pulmonary complain. of which yielded Aspergillus growth while 49 (15.7%) yielded no growth. Six patients (4.31%) were presented with tuberculosis. All of the patients have had a history of chronic bronchitis, pneumonia, cancer, suffering from Chronic Obstructive Pulmonary Disease (COPD) or have other form of pulmonary disease.Aspergillus isolates were obtained from two types of specimens, of which 47 isolates (33.8%) were obtained from Broncheoalveolar lavage (BAL) and the remaining 92 isolates (66.1%) were obtained from sputum.Aspergillus spp. were the predominant microorganism among the pathogenic fungi obtained by culture. 10 species of Aspergillus were identified, A.niger 71 (48.9%), A. flavus 71 (51%), A. fumigatus 16 (11.5%), A. terreus 40 (28.7%), A. oryzae 1 (0.71%), A. tumorous 4 (2.8%), A. candidus 9 (6.4%), A. tamari 3 (2.15%), A. parasiticus 3 (2.15%) and 1one isolate A. versicolor (2.15%).A. flavus, A. niger and A. terreus were the most frequent isolated species followed by A. fumigatus in the fourth place .Antibiotic susceptibility (MIC test) of 10 different Aspergillus isolates were performed using Epsilometer (E-test) technique. The following antifungal agents were used in the test: Caspofungin (CAS),Itraconazole (ITC), Posaconazole (POS) and Voriconazole (VO). The study results showed VO and CAS were having full inhibitory activity (100%) against the tested Aspergillus spp., while in case of ITC and POS some resistance were observed, (50% and 75% respectively).

Key words: Aspergillosis, pulmonary,Antifungals , Susceptibility testing ,MIC, E-test

Introduction

Aspergillus infections are among the most feared opportunistic infections in humans because they are capable of causing several distinct pulmonary diseases.[1] *Aspergillus* spp. is the main causal agent for fungal respiratory infections in the critically ill patient comes in second place after the fungi from the order Mucorales. [2]

The *Aspergillus* genus contains many species and these are ubiquitous in our environment. They do not form part of the normal flora. Their spores are regularly inhaled without harmful consequences, but some species, notably *A. fumigatus*, are able to cause a range of diseases, including 1- Allergic bronchopulmonary aspergillosis (ABPA), which is, as its name suggests, an allergic response to the presence of *Aspergillus* antigen in the lungs and occurs in patients with asthma. ABPA occurs in some 10% of cystic fibrosis patients, 2- Aspergilloma in patients with pre-existing lung cavities or chronic pulmonary disorders. *Aspergillus* colonizes a cavity and grows to produce a fungal ball, a mass of entangled hyphae, and 3- Disseminated disease in the immunosuppressed patient when the fungus spreads from the lungs.[3]

Aspergilloma occurs when the conidia that been inhaled enter a pre-existing cavity, germinate, and produce abundant hyphae in the abnormal pulmonary space. Those patients with previous cavitary disease (e.g. tuberculosis, sarcoidosis, emphysema) are at risk. [4] Nowadays, Voriconazole is consider the first choice for treatment of aspergillosis; amphotericin B and echinocandins (mainly caspofungin) used to treat this infection as well [5, 6]. An emerging constrain associated with Aspergillosis therapeutics is the increasing resistance against triazole that observed in *A. fumigatus* isolates. [7, 8]. The aim of this study detection the aspergillosis occurrences with patients undergo pulmonary infection , identification Aspergillus spp. and Susceptibility testing of some antifungals by MIC and E-test.

Materials and Methods

Study design and duration

This cross sectional study was designed to assess the occurrence of *Aspergillus* species in patients with pulmonary infections. Specimen collection and analysis was carried out for four months (t November 2014 to February 2015). Specimens collected from specialized center of Chest

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and Pulmonary diseases in Hilla city/Iraq. Each specimen was cultured and examined macroscopically and microscopically.

Study Subjects

Clinical specimens were obtained from patients presented with pulmonary symptoms, such as bronchiectasis, recurrent infections (with fever and malaise, dyspnea, anorexia, weight loss, and chest pain), chronic obstructive pulmonary disease COPD, or a suspected case of lung infection.The 139 *Aspergillus* isolates were gathered from 70 male (50.3%) and 69 female (49.6%), Mean age of the patients was 43.68 ± 10 years; with a wide-ranging age varied from 1 day to 90 years.

Specimens Collection

A total of 311 sputum samples collected from 311 patients presented with pulmonary complain.The patients were diagnosed clinically by specialists physician and were tabulated according to their age, gender and presence of Tuberculosis infection or other type of pulmonary disease. Seventeen 17 isolates (12.23%) were obtained from Broncheoalveolar lavage fluid (BAL) and 122 isolates (87.7%) were obtained from sputum.The 139 *Aspergillus* isolates were gathered from 70 male (50.3%) and 69 female (49.6%), Mean age of the patients was 43.68 ± 10 years; with a wide-ranging age varied from 1 day to 90 years. 6 patients with ongoing tuberculosis infection were all give positive *Aspergillus* isolates.

Specimen Processing:

Mycological analysis

The sputum specimens were transported by scow-capped cups to the microbiology laboratory and each specimen was inoculated using direct method of inoculation by streaking on two general media namely Sabouraud Dextrose agar and Malt extract agar, and by pouring on third medium namely Potato dextrose agar, Then incubated at 25 °C for 2-7 days. [9]

Antifungal Suseptibility Testing

Antifungal susceptibility by E-test method was done on non-supplemented MHA according to the method described in M51-A and M38-A2 CLSI documents. E-test gradient strips of Posaconazole (POS), itraconazole (ITC), voriconazole (VO), and caspofungin (CAS) were obtained from Liofilchem Lab, Italy. The concentration gradient for each drug ranged from 0.002 to 32 µg/ml. The strips were stored frozen on –20°C until they were used in the study. The E-test was performed by following the manufacturer's instructions. Each solidified medium was inoculated by dipping a nontoxic (latex-free) sterile swab into the respective undiluted stock inoculum suspension and evenly streaking it in three directions over the entire surface of a 150-mm petri plate containing 60 ml of medium. The plates were incubated at 35°C, and the MICs were determined following incubation after 24 h and results were confirmed by second reading after 48 h.

Results and Discussion

A total 311 specimens were collected from patients presented with pulmonary complain.Out of which 139 (44.6%) yielded *Aspergillus* growth while 49 (15.7%) yielded no growth. *Aspergillus* isolates were obtained from two types of specimens; 17 isolates (12.23%) obtained from Broncheoalveolar lavage fluid (BAL) and 122 isolates (87.7%) were obtained from sputum.

Table 1.*Aspergillus* species isolated from Bronchoalveolar lavage fluid and sputum

***Aspergillus* species**	**NO. of isolates**	**%**
A. niger	68	48.9
A. flavus	71	51
A. fumigatus	16	11.5
A.terreus	40	28.7
A.oryzae	1	0.71
A.tumorous	4	2.8
A.candidus	9	6.4
A. tamari	3	2.15
A.parasiticus	3	2.15
A. versicolor	3	2.15
Total	**139**	**100 %**

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The 139 *Aspergillus* isolates were gathered from 70 male (50.3%) and 69 female (49.6%), Mean age of the patients was 43.68 ± 10 years; with a wide-ranging age varied from 1 day to 90 years. 6 patients with ongoing tuberculosis infection were all given positive *Aspergillus* isolates.In the present study, 10 species of *Aspergillus* were identified; *A.niger* 71 (48.9%), *A. flavus* 71 (51%), *A. fumigatus* 16 (11.5%), *A. terreus* 40 (28.7%), *A. oryzae* 1 (0.71%), *A. tumorous* 4 (2.8%), *A. candidus* 9 (6.4%), *A. tamari* 3 (2.15%), *A. parasiticus* 3 (2.15%) and 1 isolate *A. versicolor* (2.15%), (Table 1.) Some specimens had grown mixed *Aspergillus* culture (63/139, 45.32%) therefore the total number of positive *Aspergillus* isolates (N=139) does not equal the total number of the individual isolates obtained (N= 218).The creditability of direct microscopy in the diagnosis of fungal infection in sputum smears have been proven. In a study,all of the 27 (100%) samples that were positive for *Aspergillus* spp. by direct sputum microscopy were positive with culture.In contrary, examination by direct microscopy failed to detect three samples, which were later found to be positive with culture. Direct microscopy is therefore 90% sensitive in detecting *Aspergillus* spp. in sputum smears. [10]

Other types of fungi beside *Aspergillus* were also isolated, these include, *Penicillium*, *Cladosporium*, *Candida*, *Acremonium*, *Bipolaris*, *Rhizopus*, *Mucor*, *Alternaria*, *Stachybotrys*, *Ulocladium*, *Fusarium*, *Blastomyces*, *Geotrichum*, *Stemphylium*, *Trichoderma*, *Streptomyces*, Mycelia sterilia and *Pythium*. Some fungal isolates were considered as contamination and therefore discarded.

Aspergillus spp. may be responsible for important clinical events from saprophytic colonization of the airways to rapidly invasive and life-threatening disseminated diseases, depending on the host immune status and the presence of underlying lung disease.[11]

The result of this study showed that *Aspergillus* spp. were the predominant microorganism among the pathogenic fungi. The results displayed a semi-equal incidence in males and females, with the isolation of mixed *Aspergillus* spp. from a single patient. The microorganism was diagnosed based on microscopic and cultural features.

In this study, it has been found that *A.flavus*, *A. niger* and *A. terreus* are the most frequent isolated species followed by *A. fumigatus* in the fourth rank (51%, 48.9%, 28.7% and 11.5%), respectively. It was found that themost frequent species of *Aspergillus* isolated were found to be *A. flavus*, *A. terreus*, and *A. niger* followed by *A. fumigatus* as the most frequent species that cause clinical disease. [12, 13]

Another study results showed (107) isolates were belonging to the genus *Aspergillus* which form (28.08%) of the total (381) isolates. The level of species belonging to the genus *Aspergillus* spp. record *A. fumigatus* as the one having highest percentage of frequency (29.9%), followed by the fungus *A. niger* (28.9%), as well as the isolated species *A. flavus* and *A. terreus* and *A. nidulans* (18.7%, 12.14%, 2.8%), respectively.[14]Several authors found that the four most frequently isolated *Aspergillus* species as a causative agent in the pulmonary infections are *A. fumigatus*, *A. flavus*, *A. niger* and *A. terreus*. [15, 16, 10]

However,in other studies the leading cause of Invasive Aspergillosis of the lungs is *A. fumigatus* (85%), followed by *A. flavus* (5 to 10%) and *A. terreus* (2 to 10%) [18- 21]. Where it has been foundthat *A. niger* (2 to 3%), *A. nidulans*, and *A. ustus* are only rarely isolated [20, 22]. In the previous reviews, *A. niger* was considered as a non-probable cause of pulmonary Aspergillosis. *A. fumigatus*was responsible for more than 90% of invasive disease in some reviews, with *A. flavus*, *A. terreus*, and *A. niger* responsible for the majority of remaining invasive aspergillosis cases, *A. niger* isolates was mostly accounted as contamination. [23]This leaves usto consider*A. flavus*, *A. fumigatus* and *A. niger* as three most probable cause of pulmonary Aspergillosis.

In *Aspergillus* infections, the lung continues to represent the most frequently involved site. Pulmonary infection are a phenotypical representation of interaction between lowered defense mechanisms in the host and the virulence of the fungus. [24]

Table 2. Categorization of the tested patients by age, gender and incidence of tuberculosis

Age Group	Males	Females	Total	Number of Pts. with TB
< 10	**4**	**4**	**8**	
10-20	**13**	**15**	**28**	

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20-30	**31**	**34**	**65**	**1**
30-40	**31**	**30**	**61**	**3**
40-50	**27**	**26**	**53**	**1**
50-60	**25**	**21**	**46**	
60-70	**23**	**17**	**40**	**1**
70-80	**6**	**1**	**7**	
80-90	**1**	**1**	**2**	
90-100	**1**		**1**	
Total	**162**	**149**	**311**	**6 cases**

This study result showed that patients with pulmonary diseases and patients with lower immune status are mainly at risk of infection by pathogenic *Aspergillus* spp. and the fact that pulmonary abnormalities are a predisposing factor for the fungal infection.Table 2.display the age and gender variation among the patients selected in this study, and shows the incidence of Tuberculosis infection in the study and its correlation with the showed age period. The six6 patients whose presented with tuberculosis were having a concomitant Aspergillosis infection (6/139,4.31%). Moreover, along with those 6 patients with proven tuberculosis, almost all of the patients (211/311, 67.84%) have had a history of chronic bronchitis, pneumonia, cancer, undergone COPD attack or suffering from COPD during the time when the sample has been taken or have other form of pulmonary disease. Others have a suspected case of pneumonia with or without immunocompromisation.

In a single study, nine 9 (4.5%) patients infected with both infections (Tuberculosis and Aspergillosis). Among the nine9 patients who were infected with *Aspergillus* sp. and TB, six 6 were infected with *A. fumigatus*, two 2 with *A. niger* and one 1 with *A. flavus*. None of the patients was infected with *A. terreus*[10].Previous tuberculosis (either classical or atypical) was the most commonly identified primary underlying condition (38/126, 30.2%). The second common, primary underlying condition was ABPA (15/126, 11.9%). However, all of the 232 underlying conditions identified for 126 Chronic Pulmonary Aspergillosis (CPA) cases, COPD/emphysema was the most common (42/126, 33.3%). Classical tuberculosis and non-tuberculous mycobacterial infection were also very common (41/126, 32.5%), as were pneumothorax (21/126, 16.7%), pneumonia (28/126, 22.2%) and asthma (13/126, 10.3%).[25]

The importance of tuberculosis in the development of aspergillosis is supported by the results from studies of tuberculosis patients. These studies have found that out of 544 patients who were left with a residual cavity ≥2.5 cm 1 yr after cured tuberculosis, 36% had positive *Aspergillus* antibodies and 22% had radiological aspergillomas after 3 yrs., these results were introduced at 1968 and 1970[26].It implies that 8–12% patients who recover from classical tuberculosis develop CPA over 4 yrs.studies reported a substantial increase in diagnosed acute invasive pulmonary aspergillosis (IPA) in COPD, which has a 95% mortality rate.[27, 28]

Susceptibility testing

Due to an increase in the numbers of patients at-risk for fungal infections, such as patients with lowered immune status and cancer patients who receive chemotherapy, the incidence of systemic mycoses has increased. One of the most common fungal pathogens is the species of *Aspergillus*. Since fungal infections in hospitals are much less frequent than microbial infections, susceptibility tests for fungi are not routinely performed[29].In the present study, ten 10 *Aspergillus* spp. isolates were tested by using E-test. Four 4 Antifungal agents have been used. Three of which belong to the class Azole: Itraconazole (ITC), Posaconazole (POS) and Voriconazole (VO), while the fourth antifungal agent belonged to the class Echinocandins: Caspofungin (CAS).

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There was a tendency of agar- based method to produce false susceptible results after 24 h which can be explain, e.g., as being due to the shift of the interpretive categories in some *Aspergillus* strains (such as *A. terreus*) when comparing reading after 24h and 48h[30].Therefore, in case of *A. terreus* the first reading at 24h was considered while in the case of the remaining *Aspergillus* spp. the second reading (at 48h) was considered.

The E-test was carried on non-supplemented MHA. Antifungal agents showed good activity against *Aspergillus* spp. except of some forms of resistance which have appeared in some isolates. Even though the antifungals tested are not in use nationally and it was the first time the fungus exposing to them. Several authors mentioned that there were no difference in MIC or MEC values at 24 hr and 48 hr, therefore we suggest E-test to be done on non-supplemented MHA in place of RPMI 1640 agar with 2% glucose and MOPS, which is also the common media available in most of the laboratories.[31]

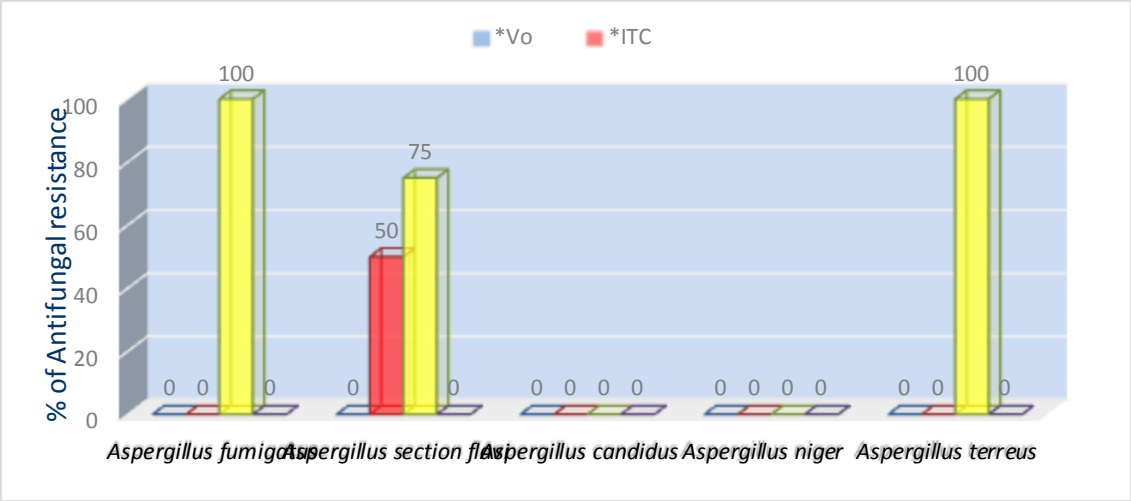

Figure 2. Resistance of Aspergillus isolates to different antifungal agents using E-test. *VO: Voriconazole, *ITC: Itraconazole, *POS: Posaconazole, *CAS: Capsofungin.

In the present study, resistance to ITC was seen in 20% of isolates, where's 30.6% of isolates were resistant to ITC[29]. It was found that echinocandins exhibited a good activity against *A. fumigatus* isolates. However, azole agents had different activity *in vitro*. The first isolate was susceptible *in vitro* to ITC, VO (MIC ≤ 1 mg/L), and POS (MIV value ≤ 0.25 mg/L). The second isolate, obtained after VO therapy, was resistant in vitro to ITC, VO and POS [32].Resistance to ITC is usually associated with a reduction in POS susceptibility, predictably because the 2 drugs are structurally similar[33].This study test results show resistance to ITC and POS by *Aspergillus section flavi*. Isolates with reduced susceptibility to ITC are frequently cross-resistant to other triazoles, and specific testing is recommended [34].The MIC results obtained by E-test methods demonstrated that POS was very active against all *Aspergillus* spp. (all susceptible at MIC <0.25 µg/ml). All isolates of *Aspergillus* spp. were inhibited with <0.5 µg/ml of VO. [35]

Table 2. Susceptibilities of *Aspergillus* isolates to Voriconazole, Itraconazole, Posaconazole and Caspofungin by E. test

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Aspergillus Species (no. of isolates)	Antifungal agent	*MIC (µg/ml)	
			E. test
		Range	90%
A. fumigatus (3)	Voriconazole	0.250-1.000	0.190
	Itraconazole	0.250-2.000	0.500
	Posaconazole	0.010-4.000	4.000
	Caspofungin	0.250-1.000	0.125
Aspergillus section flavi (4)	Voriconazole	0.064-0.640	0.250
	Itraconazole	0.250-0.500	0.500
	Posaconazole	0.500-4.000	4.000
	Caspofungin	0.250-1.000	0.032
A. terreus (1)	Voriconazole	0.120-1.000	-
	Itraconazole	0.010-0.500	-
	Posaconazole	0.010-0.060	-
	Caspofungin	0.250-1.000	-
A. niger (1)	Voriconazole	0.064-0.190	-
	Itraconazole	1.500-32.00	-
	Posaconazole	0.250	-
	Caspofungin	0.032	-
A. candidus(1)	Voriconazole	0.061-0.064	-
	Itraconazole	0.064-0.250	-
	Posaconazole	0.032-0.250	-
	Caspofungin	0.032	-

*MIC: Minimum inhibitory concentration

These results demonstrate the excellent efficacy of voriconazole against *Aspergillus* species and suggest that voriconazole may be the treatment of choice in invasive aspergillosis caused by *A.fumigatus* and *A. flavus*. Voriconazole was found to have MIC of ≥ 2 µg/ml in 9.1% *Aspergillus* isolates [28]. Voriconazole of an MIC of ≥ 4 µg/ml was reported in 4.9% of *A. flavus* and considered as sensitive.[35]

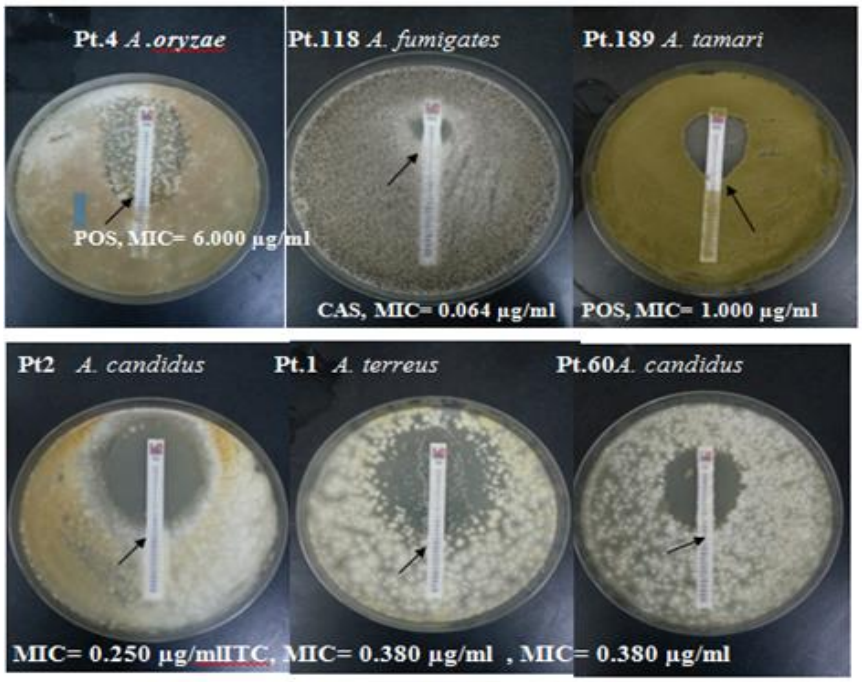

Figure 3.E-test gradient strips of different antifungal agents showing susceptibility of *A. fumigatus*, *A. alliaceous, A. tamari, A. candidus, A. terreus* (Isolates No: 4, 118, 189, 302, 18 and 60).

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However, resistance of *A. fumigatus* to the azoles was reported to vary from a high of 52% and 38% with ITC and ravuconazole, respectively, to a low of 11% with VO. As this study shows high resistance of *A. fumigatus* to the azole agent POS [36]. All species showed susceptibility to VO. For ITC, 93 (86.1%) species were susceptible, with 15 (13.9%) resistant. [29]

In conclusion, The fungus *Aspergillus* was found to be the predominant fungal pathogen isolated from patients with pulmonary diseases, notably from patients with COPD and pulmonary TB. *A.flavus*, *A.niger* and *A. terreus* were the most frequent isolated spp. followed by *A. fumigatus* in the fourth place. All of the tested *Aspergillus* spp. were displaying marked resistance against amphotericin B, and traditional Azoles, (most notably to fluconazole. For the newer azoles, voriconazole and caspofungin were having full inhibitory activity against the tested *Aspergillus*.

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