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Antifungal Susceptibility Patterns of *Aspergillus* species isolated from patients with Pulmonary diseases in Iraq

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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 was to determine the prevalence of *Aspergillus* species in patients with pulmonary pneumonia and susceptibility testing to the most commonly used antifungal agents. A total of 311 specimens were collected from patients presented with the pulmonary complaint. Out of which, 139 (44.6%) yielded *Aspergillus* growth. *Aspergillus* isolates were obtained from Bronchoalveolar lavage and sputum. Ten species of *Aspergillus* were identified, *A. flavus*, *A. niger,* and *A. terreus* were the most frequently isolated species. Antifungal susceptibility of 10 different *Aspergillus* spp. isolates were performed using E-test. Voriconazole and caspofungin were having full inhibitory activity (100%) against tested *Aspergillus* spp., while some resistance was observed against itraconazole and posaconazole (50% and 75% respectively). *Aspergillus* was found to be the predominant fungal pathogen isolated from patients with pulmonary diseases, notably from patients with COPD and pulmonary TB.

INTRODUCTION

Aspergillus infections are among the most feared opportunistic infections in humans because they are capable of causing several distinct pulmonary diseases (Krel *et al*., 2014). *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 (Garnacho-Montero *et al*., 2013).

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 (Goering *et al*., 2013).

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 (Brooks *et al*., 2014). Nowadays, Voriconazole is considered the first choice for treatment of aspergillosis; amphotericin B and echinocandins (mainly caspofungin) used to treat this infection as well (Walsh *et al*., 2008; Mikulska and Viscoli, 2011). An emerging constraint associated with Aspergillosis therapeutics is the increasing resistance against triazole that observed in *A. fumigatus* isolates (Denning *et al*., 2011; Snelders *et al*., 2011).

The aim of this study was to determine the prevalence of *Aspergillus* species in patients with pulmonary pneumonia, and antifungal susceptibility testing of these clinical fungal isolates by E-test technique.

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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 (1st November 2015 to 30th February 2016). Specimens collected from the specialized center of Chest and Pulmonary diseases in Hilla city/Iraq. Each specimen was cultured and examined macroscopically and microscopically.

Study subjects

Clinical specimens were obtained from patients (70 male and 69 female), 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. Mean age of the patients was 43.68 ± 10 years; with a wide-ranging age varied from 1 day to 90 years. The patients were diagnosed clinically by specialists and were tabulated according to their age, gender, and presence of Tuberculosis infection or another type of pulmonary disease.

Ethical approval

Verbal consent was taken from each patient before sampling. Investigative standards were rigidly preserved, primarily concerning confidentiality. Moreover, this study was undisclosed, the participation of patients was optional, and verbal consent was received before data uptake process was started.

Specimen collection and mycological analysis

A total of 311 specimens were collected from patients presented with the pulmonary complaint. Specimens included Bronchoalveolar lavage fluid (BAL) and sputum. The specimens were transported by screw-capped cups to the Microbiology lab and each specimen was inoculated by streaking on two general culture media (Sabouraud Dextrose agar-supplemented with chloramphenicol and Malt extract agar), and by pouring on third medium namely Potato dextrose agar, Then incubated at 25°C for 2-7 days (Forbes *et al*., 2014).

Antifungal susceptibility testing

Antifungal susceptibility by E-test method was done on non-supplemented Muller Hinton Agar (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 at −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 read at 24h or 48h as the concentration of drug that elicited 100% inhibition of growth (posaconazole, itraconazole, voriconazole) or as the minimum effective concentration (MEC, caspofungin).

There was a tendency of an agar-based method to produce false susceptible results after 24 hours which can be explained, e.g., as being due to the shift of the interpretive categories in some *Aspergillus* strains when comparing reading after 24h and 48h (Buchta *et al*., 2008). 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 was considered.

RESULTS AND DISCUSSION

Out of total 311 specimens collected, 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 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%). Six patients with ongoing tuberculosis infection were all given positive *Aspergillus* isolates.

Ten species of *Aspergillus* were identified (Table 1). *A. flavus*, *A. niger,* and *A. terreus* were the most frequently isolated species followed by *A. fumigatus* in the fourth place (51%, 48.9%, 28.7%, and 11.5%), respectively.

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

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

Some specimens had grown mixed *Aspergillus* culture (63 isolates (45.32) out of 139) 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 has been proven.

In a study, all of the 27 (100%) samples that were positive for *Aspergillus* spp. by direct sputum microscopy and 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 (Njunda *et al*., 2012).

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 (Latgé *et al*., 2001).

Several authors found that the most frequent species of *Aspergillus* associated with clinical disease cases were *A. flavus*, *A. terreus*, and *A. niger* followed by *A. fumigatus* (Malik *et al*., 2009; Shujat *et al*., 2014).

Another study showed that 28.08% of total 381 isolates were belonging to the genus *Aspergillus*. *A. fumigatus* was the one having highest percentage of frequency (29.9%), followed by *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 (Aziz, 2014). It was 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* (Njunda *et al*., 2012; Barberan *et al*., 2012; Diba *et al*., 2007).

Various studies reported that *A. fumigatus* is more prevalent in the Western countries, while A. *flavus* is more prevalent in the Middle East and Asia as the leading cause of invasive aspergillosis of the lungs (Patterson *et al*., 2000; Groll and Kolve, 2004; Chamilos and Kontoyiannis, 2005; Singh and Paterson, 2005; Pfaller *et al*., 2006). However, it has been found that *A. niger*, *A. nidulans*, and *A. ustus* are only rarely isolated (Pfaller *et al*., 2006; Shao *et al*., 2007). *A. niger* was considered as a non-probable cause of pulmonary Aspergillosis.

A. niger isolates were mostly accounted as contamination (Patterson *et al*., 2000). This leaves us to consider *A. flavus*, and *A. fumigatus* as two most probable cause of pulmonary Aspergillosis.

In *Aspergillus* infections, the lung continues to represent the most frequently involved site. Pulmonary infection is a phenotypical representation of the interaction between lowered defense mechanisms in the host and the virulence of the fungus (Pagano *et al*., 2008).

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.

Results also found that six patients were presented with tuberculosis. These patients with TB were having a concomitant Aspergillosis infection (6/139, 4.31%). Moreover, along with those with proven tuberculosis, almost two-thirds 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 another form of the 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 nine 9 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* (Njunda *et al*., 2012). 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%).

Antifungal susceptibility testing

Since fungal infections in hospitals are much less frequent than other microbial infections, susceptibility tests for fungi are not routinely performed in some diagnostic laboratories (Badiee *et al*., 2012). In the present study, ten 10 *Aspergillus* spp. isolates were tested by using E-test against four antifungal agents ITC, POS, O, and CAS).

The E-test was carried on non-supplemented MHA (Figure 1). Antifungal agents showed good activity against *Aspergillus* spp. except for some forms of resistance which have detected in some isolates even though the antifungals tested are not in use nationally and it was the first time the fungus exposing to them (Table 2). A group of authors mentioned that there was no difference in MIC or MEC values at 24 hr and 48 hr, therefore we suggest E-test 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 (Gupta *et al*., 2015).

In the present study, resistance to ITC was seen in (20%) of isolates. Badiee *et al*. (2012) reported that 30.6% of *Aspergillus* species isolates were resistant to ITC when tested by E-test. 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 (MIC value ≤ 0.25 mg/L). The second isolate, obtained after VO therapy, was resistant *in vitro* to ITC, VO, and POS. Mellado *et al*. (2013) reported that pulmonary *A. fumigatus* isolates recovered from Spanish patient were multiple triazoles resistant.

Resistance to ITC is usually associated with a reduction in POS susceptibility, predictably because the 2 drugs are structurally similar (Rodriguez-Tudela *et al*., 2008). This study showed 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 (Arendrup *et al*., 2012). 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 (Rudramurthy *et al*., 2011).

These results demonstrate the excellent efficacy of VO against *Aspergillus* species and suggest that VO may be the treatment of choice in invasive aspergillosis caused by *A. fumigatus* and *A. flavus*. VO was found to have high-MIC in 9.1% *Aspergillus* isolates (Guinea *et al*., 2010). VO of a low-MIC was reported in 4.9% of *A. flavus* and considered as sensitive (Rudramurthy *et al*., 2011).

However, Kaufman *et al*. (2004) found that 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. They also reported that high resistance of *A. fumigatus* to the azole agent POS was detected.

Table 2: Susceptibility of *Aspergillus* isolates to the most commonly used antifungal agents by E. test.

Aspergillus Species (no. of isolates)	Antifungal agent	MIC/MEC (µg/ml)	
		Range	E. test
			90%
Aspergillus 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
****Aspergillus terreus (1)***	Voriconazole	0.120-1.000	-
	Itraconazole	0.010-0.500	-
	Posaconazole	0.010-0.060	-
	Caspofungin	0.250-1.000	-
Aspergillus niger (1)	Voriconazole	0.064-0.190	-
	Itraconazole	1.500-32.00	-
	Posaconazole	0.250	-
	Caspofungin	0.032	-
Aspergillus candidus (1)	Voriconazole	0.061-0.064	-
	Itraconazole	0.064-0.250	-
	Posaconazole	0.032-0.250	-
	Caspofungin	0.032	-

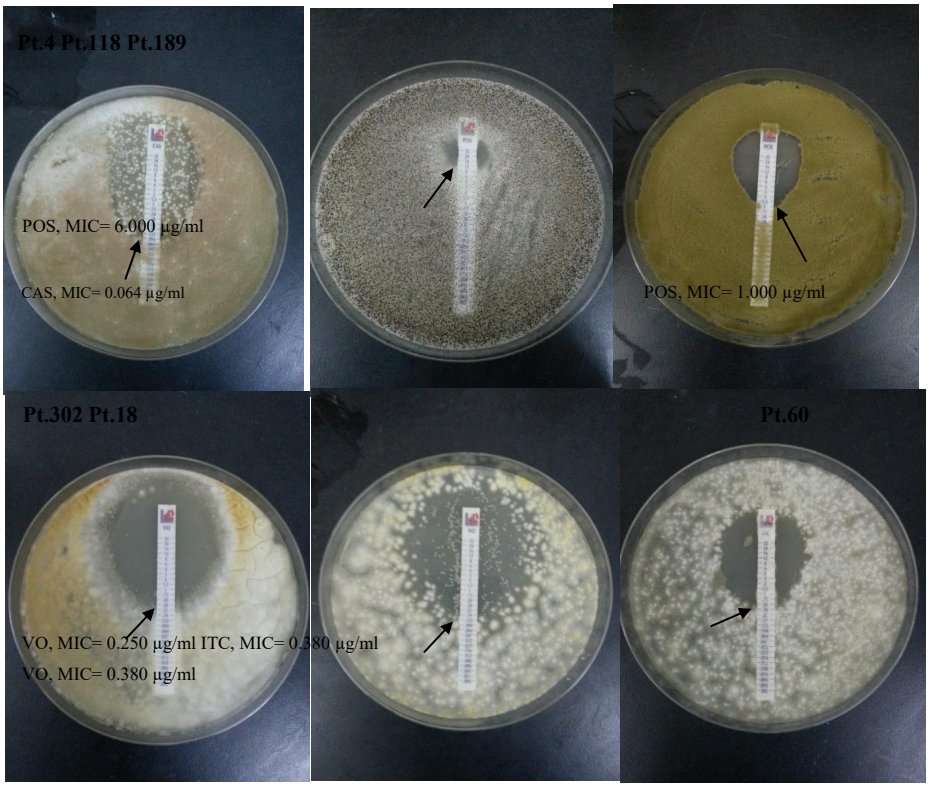

Fig. 1: E-test gradient strips of different antifungal agents and different *Aspergillus* species (Isolates No: 2 and 118).

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. *Aspergillus 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* species were displaying marked resistance against traditional Azoles, (most notably to fluconazole. For the newer Azoles, VO and CAS were having full inhibitory activity against the tested Aspergilli.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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