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(RESEARCH ARTICLE)

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Aspergillus fumigatus identification and antibiotic sensitivity in Iraqi patients

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Abstract

Pulmonary aspergillosis is a severe infection in immunocompromised caused by Aspergillus fungi, with *Aspergillus fumigatus* being the most frequently cultured of this genus, although A. flavus, *A. niger, A. terrus* also include If left untreated, invasive aspergillosis can have mortality approaching 100%.

A total of 105 samples were collected from TB patients with symptom of lung infection . These sputum samples were obtained from patients treated in Public health department Chest and Respiratory diseases clinic, from February 2023 to April 2023. All samples were transferred to the laboratory for culturing to isolation and identification fungi after labeling each sample with questionnaire form. Lacto phenol cotton blue staining method was used in identification of fungal isolates, fungi isolated by inoculating sample on culture media such as sabouraud dextrose agar (SDA), and disc diffusion.

The sample mean ± standard deviasion (SD) of age was 48.5±14.2 years (ranged 9-75 years). Male to female ratio was 2:1.5, age groups number and percentage were 32(30.5%), 13(12.4%), 14(13.3%), 14(13.3%), 16(15.2%) and 16(15.3%) in the age groups <=29, 30-39, 40-49, 50-59, 60-69 and >=70. Laboratory investigations indicated that 40 (38.1%) of the total samples diagnosed with a fungus species, 16 (15.3%) of them present with Candida species followed by 14 (13.3%) with *A.fumigatus*, 5 (4.8%) with *A. niger*, 3 (2.8%) with *A.terreus* and 2 (1.9%) *A.flavus*, while negative results were indicated in 65 (61.9%) of collected samples. *A.fumigatus*. isolates were subjected to susceptibility testing using different antifungals. The antifungal drugs NYS, KEA, CMZ, ICZ and PCZ were the most effect. Resistant outcomes were reported in FCZ**,** while intermediate results were showed in MIC and AMB. The results showed an increase in the prevalence of *Aspergillus fumigatus* in Iraqi patients. This prevalence may be alarming on public health.

Keywords: *Aspergillus fumigatus*; Respiratory; Antibiotics; Iraqi patients; Antifungal drugs

1. Introduction

Pulmonary aspergillosis is a severe infection in immunocompromised caused by Aspergillus fungi, with *Aspergillus fumigatus* being the most frequently cultured of this genus, although *A. flavus*, *A. niger, A. terrus* also include (1). As the Aspergillus is an airborne easily transmissible infection and it causes severe invasion (2). Aspergillus species considered a one of the main causes of life-threatening infections in hospitalized patients with significant morbidity and mortality rates even when appropriately diagnosed and treated (3). If left untreated, invasive aspergillosis can have mortality approaching 100%. In cases of suspected invasive aspergillosis, an extensive diagnostic workup is necessary, but treatment should be initiated early to reduce morbidity and mortality (4).

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Fungal diseases kill more than 1.5 million and affect over a billion people. However, they are still a neglected topic by public health authorities even though most deaths from fungal diseases are avoidable. Recent global estimates have found 3,000,000 cases of chronic pulmonary aspergillosis, ~250,000 cases of invasive aspergillosis (5). Invasive fungal infection (5) account for about 15% of severe respiratory infections (6). age and underlying cancer were associated with higher mortality among invasive aspergillosis patients (7).

There are many risk factors for developing pulmonary aspergillosis: evidence of suppressed immunity (including infection with the human immunodeficiency virus, bone marrow and solid organ transplantation, prolonged corticosteroid use, systemic anti-biological therapies, poorly controlled diabetes mellitus and chronic renal failure), presence of structural lung disease (sarcoidosis, cystic fibrosis, chronic obstructive pulmonary disease) and factors that may accelerate fungal colonization (wider use of antimicrobials) (8, 9).

Aspergillosis can be divided into three distinct clinical categories: allergic aspergillosis, saprophytic colonization, and invasive aspergillosis. Recent study reported that the lung was the most common site of infection (10). Recent study reported IPA has a high potential to be overlooked due to its nonspecific clinical symptoms and radiological findings. Clinicians should suspect IPA not only in immunocompromised patients but also in oncological patients presenting with lung infection symptoms, especially those with lung cancer (11). past study reported Patients with solid tumors had better antifungal therapy response and lower 12-week IPA-attributable mortality than did those with hematologic malignancies (12). Aspergillus species are developing resistance mechanisms to the treatment options that are currently available. Since the mortality rate, is as high as 50% in treated individuals, prophylaxis may be a viable option for IA infections (13). Species can be identified by colony morphology and microscopic characterization of conidia and conidiophores or by PCR (14).

2. Material and methods

2.1. Sample collection

Samples collected from Respiratory Patients were attending or Chest and Respiratory diseases Consulting Clinic. The patients have lung infection were diagnosis by Physician. questioner form was containing many information taken from patients such as (name of patients, age, gender, type of TB Positive or Negative). A total of 105 samples were collected from TB patients with symptom of lung infection. These sputum samples were obtained from patients treated in Public health department Chest and Respiratory diseases clinic, from February 2023 to April 2023. All samples were transferred to the laboratory for culturing to isolation and identification fungi after labeling each samples with questionnaire form.

2.2. Preparation of Culture Media

2.2.1. Sabouraud's1Dextrose Agar (SDA)

The media was created by dissolving 65 grams of standard in one liter of distilled water, followed by the addition of 0.05 grams of chloramphenicol per liter of medium. after being autoclaved for 15 minutes at 121 °C, pour the medium in a sterile petri dish, and left it to solidify and dry to be suitable for culturing. Inoculation of the samples (swab sample) and incubated for 10 days at 25˚C and 24-48h at 37˚C under aerobic conditions.

2.2.2. Brain Heart Infusion Broth (BHI)

This medium was made by dissolving 52g in one liter of distilled water, then boiling1it to thoroughly dissolve it. Then, it is poured into a sterile screw-capped1 tube and1autoclaved at 121 °C for 151minutes (according to the manufacturer's company instruction). The1medium was employed to activate the isolates.

2.2.3. Mueller Hinton Agar (MHA)

Mueller Hinton Agar was1prepared1from a dried infusion of1beef, acid hydro lysate of casein to provide1amino acids1and other1nitrogenous substances, minerals, nutrients to support the growth of microbes, such as vitamins, carbon, and other elements, Soluble starch was1added to absorb any toxic1substances which may be1present in the1medium. When starch was hydrolyzed during autoclaving, a little amount of dextrose was produced as an energy source. Agar was used as the solidifying agent Suspend 38.0 grams in 1000 ml purified/ distilled water. Heat to boiling was done to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes (according to the manufacturers company instruction). After autoclaving, pour the medium in sterile petri dish, and left it to solidify and dry to be suitable for culturing **(15).**

2.3. Lacto phenol Cotton blue staining method

Lacto phenol Cotton blue (LPCB) staining method considered one of the most useful identification techniques used in the preparation of slides for microscopic examination of fungi (hyphae or yeast). Fungal elements are stained intensely blue. The lacto phenol cotton blue (LPCB) wet mount preparation was the most common way of staining and viewing fungus, and it's easy to do. There are three ingredients in the mixture: phenol, which kills any living organisms; lactic acid, which maintains fungal structures; and cotton blue, which colors the chitin in fungal cell walls **(16).**

2.4. Identification of Fungal Isolates

Fungi isolated by inoculating sample on culture media such as sabouraud dextrose agar (SDA), as three replicates for one sample, and incubated at (28 °C) for (5-10) days, and identify consistency, form, color, and revers color of the colonies (17). Isolated fungi were identified by macroscopic and microscopic examination and molecular diagnosis by PCR and sequencing methods. Microscopic examination for identifying characteristics of conidia, stipes, and length, conidia size, vesicles form and seriation, Microscopic characteristics are Conidial, stipes, and length, shape, roughness, vesicles form and seriation, conidia size, metula covering, as well as colony parameters such as diameter after (7 days), mycelia, color of conidia (18); (19).

2.4.1. Tape technique

- A small amount from lacto phenol cotton blue stain was putted on slid
- From roll of tape 2 cm was cut and attached one end to forceps and then the colony was touched by tape
- The tape was placed on LPCB in the center of slid
- The slid was examined by microscope **(20).**

2.5. Antifungal Susceptibility Testing

2.5.1. Disc Diffusion Test

Standard methods for antifungal test have been used in this study, by approved methods for testing of both yeast and filamentous fungi. The antifungal susceptibility of the isolates was interpreted as sensitive (S), intermediate (I), and resistant (R).

2.5.2. Inoculum Preparation

Inoculum was prepared by picking1distinct colonies of like morphological type were selected from be subculture on to Sabouraud's dextrose agar to1ensure purity and viability. The incubation temperature is 25°C for molds and 37°C for yeasts. Colonies were suspended in 3 mL of sterile1normal saline. Turbidity1was adjusted to a 0.51McFarland standards for yeasts and 1.0 McFarland standards for molds (ready to use).

2.5.3. Application of Discs

Antifungal discs were placed on the surface of the inoculated agar plate by using sterile forceps. Each of the1discs should be placed flat onto the agar surface to achieve total contact. They have to be uniformly scattered and no1closer than 24 mm apart from center to1center. In an incubator, the 1plates were inverted and put within 151minutes1after the discs were1applied.

3. Results

3.1. Descriptive data of subject included in this study

This study included a total of one hundred five samples (n = 105) of patients suspected for fungal infection. The sample mean ± standard deviation (SD) of age was 48.5±14.2 years (ranged 9-75 years). Male to female ratio was 2:1.5, age groups number and percentage were 32(30.5%), 13(12.4%), 14(13.3%), 14(13.3%), 16(15.2%) and 16(15.3%) in the age groups <=29, 30-39, 40-49, 50-59, 60-69 and >=70, table (1).

Table 1 Demographic characteristics of patients included in this study

		Count	%
Gender	Male	65	61.9%
	Female	40	38.1%
Age groups	<=29	32	30.5%
	30-39	13	12.4%
	40-49	14	13.3%
	50-59	14	13.3%
	60-69	16	15.2%
	>=70	16	15.3%

3.2. Distribution of fungus species

The laboratory investigations indicated that 40 (38.1%) of the total samples diagnosed with a fungus species, 16 (15.3%) of them present with Candida species followed by 14 (13.3%) with *A. fumigatus*, 5 (4.8%) with *A. niger*, 3 (2.8%) with *A.terreus* and 2 (1.9%) *A.flavus*, while negative results were indicated in 65 (61.9%) of collected samples (Table 2).

Table 2 Distribution of fungus species in current study

Pathogen	Frequency	Percent
Candida	16	15.3%
A. fumigatus	14	13.3
Niger	5	4.8
A. terreus	3	2.8
A. flavus	2	1.9
Negative results	65	61.9%
Total	105	100.0

3.3. Antifungal sensitivity of *A. fumigatus* determination using disk diffusion

Table 3 Results of antibiotic sensitivity test

Antibiotics	R	I	S
NYS	0(0.0%)	0(0.0%)	14(100%)
KEA	0(0.0%)	0(0.0%)	14(100%)
CMZ	0(0.0%)	0(0.0%)	14(100%)
ICZ	0(0.0%)	0(0.0%)	14(100%)
PCZ	0(0.0%)	0(0.0%)	14(100%)
MIC	0(0.0%)	14(100%)	0(0.0%)
AMB	0(0.0%)	14(100%)	0(0.0%)
FCZ	14(100%)	0(0.0%)	0(0.0%)

A total of 14 *A.fumigatus*. isolates were subjected to susceptibility testing using different antifungals; Nystatin (NYS), miconazole (MIC), Clotrimazole (CMZ), Fluconazole (FCZ), Itraconazole (ICZ), Amphotericin B (AMB), Ketoconazole (KEA) and Posaconazole (PCZ). The susceptibility to antifungals for these isolates is outlined in the table (3). The antifungal drugs NYS, KEA, CMZ, ICZ and PCZ were the most effect. Resistant outcomes were reported in FCZ**,** while intermediate results showed in MIC and AMB, (Table 3) and (Figure 1).

4. Discussion

There are very few data on the burden of fungal diseases, unlike many of the Arab League countries (21). it had estimated the incidence and prevalence of the most serious fungal diseases using national, regional and international data in specific populations at risk(22). Differences in sex have a determining influence on the prevalence and incidence of many infectious diseases, including infections caused by bacteria, viruses, parasites and fungi (23). Sex-dependent host immune responses, hormone homeostasis and varying behavioural characteristics lead to distinct susceptibility and epidemiology of infectious diseases. Due to increased concern that the generalizability and reproducibility of research findings in animal models does not adequately address the standardisation of experimental set-ups, in 2017 the National Institutes of Health (NIH) announced policies that require applicants to report plans for balancing male/female ratios in animals in order to be considered for funding (24).

In current study male to female ratio was 2:1.5, this agreed to vom Steeg and Klein, (23) who showed that male predominance, in terms of the prevalence/incidence and severity, has been hypothesised for infections caused by fungi ,detailed analyses are lacking that more comprehensively investigate sex differences in fungal diseases, including in animal model studies and review the literature on sex differences in fungal diseases caused by moulds, yeast and selected endemic mycoses. In addition Bongomin et al. (25), Dotis et al., (26), Hammond et al., (27) and Yelika et al., (28) indicated that in total of 42 meta-analyses/systematic reviews on fungal infection, which were conducted between 2001 and 2022, a male predominance ranging from 51% to 93.5% .

While our data were non compatible to many studies mentioned that in addition, sex distribution in the general population is often not considered when interpreting results on sex differences, such as Eurostat, (29) as sex demographics for Europe in 2020 show percentages of 51.14% females 48.86% males, Unted Nation, (30) mentioned that 50.52% females 49.48% males in 2020 and Joseph Mathew, (31) revealed that global estimates report 49.58% females 50.42% males in 2021. Egger et al. (32) revealed that biological sex, which comprises differences in host sex hormone homeostasis and immune responses, can have a substantial impact on the epidemiology of infectious diseases, data on sex distributions in invasive fungal diseases (IFDs) are lacking. Females represented 51.2% of invasive gungal cases, mostly matching the proportions of females among the general population in the United States and Europe (>51%). In contrast, other IFDs were overrepresented in males, including invasive fungal infection (51% males). Behavioural variations, as well as differences related to biological sex, may only in part explain these findings (32). These differences in outcomes may due to the number of collected samples in each study.

In this study age group 29 represented (30.5%) of collected samples. These findings were in consistence with Loster et al., (33) revealed that the genus of fungi species and the frequency of yeast infection, wearers appear to be influenced by both age and gender. The complete infection wearers ≤50 years of age appeared to have the greatest proclivity to oral fungal infections, In every age group, the number of infection-free individuals was greater among males than females. Intermediate, intense, and abundant growth of mycosis occurred most frequently in the youngest group. (33). Ogba et al., (34) indicated that the overall prevalence of pulmonary mycoses was 36.0%, patients aged 25–34 years were at the highest risk of pulmonary mycosis (43.9%), (p = 0.00). Zhu et al. (35) reported that in total, 24 respiratory pathogens were found among the patients, and 242 (94.2 %) patients were infected with one or more pathogens. Bacterial co-infections were dominant in all patients the highest and lowest rates of infections were found in patients aged 15 and below 15, respectively. In addition, the proportion of fungal coinfections and bacterial-fungal co-infections were the highest in all cases.

While our outcomes were not the same of Sani et al. (36) recorded that pulmonary mycosis (PM) poses a great diagnostic challenge due to the lack of pathognomonic and radiological features, especially in the absence of mycology laboratory tests. Samples were processed using standard mycological staining, microscopy, sugar biochemistry, and antifungal susceptibility test protocols. Of the 216 participants, 19.9% had PTB and 73.6% had pulmonary fungal pathogens. Among the isolated pulmonary fungal pathogens, *Aspergillus fumigatus* made the highest occurrence, no significant association existed between the prevalence of PM with age and sex of participants (P < 0.05). Rafat et al. (37) and Hashemi, (38) mentioned that a total of 384 lung specimens (192 sputum samples) were obtained from symptomatic patients hospitalized in pulmonary units. Of these, 137 (35.67%) were positive in direct examination and culture. Among the 137 positive cases, most isolates were from male patients 86 (62.77%) and most of them were between 46

and 72 years .. This differences in outcomes could be attributed to the genetic or to other diseases that individuals suffering from.

The results of the current study were in consistence with John et al., (39), Balan and Viswanatha, (40) reported that common fungal agents causing secondary infection in Chronic suppurative otitis media (CSOM) are *Candida spp*., *Aspergillus spp., Rhizopus spp*., and *Penicillium spp*. Previous literature showed that among the fungal isolates, *Aspergillus* as well as *Candida* were the most widespread species responsible for otomycosis globally but other causative isolated fungi species have been belonged to genera *Penicillium*, *Fusarium*, *Mucoraceae*, *Scopulariopsis*, *Alternaria*, *Malassezia* in addition to various dermatophytes (41). Aggarwal and Jaiswal, (42) mentioned that it was identified Aspergillus species in 60% of patients. Mohammed et al., (43) and Pandey et al., (44) proved that the incidence and etiology of pulmonary fungal diseases can vary in various types of patient's hospital settings, and geographical locations. Fungi which affect immunosuppressed individuals are frequently species of *Aspergillus, Candida* and *Cryptococcus* geographically restricted agents, and newly emerging fungal pathogens.

On the other hand the results disagreed with Yahia and Alsayed, (45) revealed that *Aspergillus fumigatus was* the most common fungus in the study (n=70, 36%) followed by *Candida tropicalis* (n=28,16%). Positive fungal cultures were observed in 180 specimens (90%). Concluded that *Aspergillus fumigatus* and *Candida tropicalis* were the most prevalent isolated fungi in those patients. As *Candida* infection was reported as the most dominant pulmonary fungal diseases in patients with non-hematologic malignant tumors and in non-lung SOT recipients. Taken together, the clinicians must remain vigilant for invasive and serious pulmonary fungal diseases even to individuals who were once considered only moderately immunocompromised. This difference in results may due to age of patients included.

In this study *A.fumigatus* isolates were most sensitive to Nystatin, Ketoconazole, Clotrimazole, while Fluconazole was resist, intermediate was recorded in Amphotericin B treatment. These outcomes were agreed to Culibrk et al. (46) revealed that nystatin is a fungicide which kills conidia but cannot penetrate the cell membrane. *Aspergillus fumigatus* is an opportunistic fungal pathogen capable of causing severe infection in humans. One of the limitations in our understanding of how *A. fumigatus* causes infection concerns the initial stages of infection, notably the initial interaction between inhaled spores or conidia and the human airway. Using publicly-available datasets, we identified the Arp2/3 complex and the WAS-Interacting Protein Family Member 2 WIPF2 as being potentially responsible for internalization of conidia by airway epithelial cells. Using a cell culture model, we demonstrate that RNAi-mediated knockdown of WIPF2 significantly reduces internalization of conidia into airway epithelial cells. Furthermore, we demonstrate that inhibition of Arp2/3 by a small molecule inhibitor causes similar effects. Using super-resolution fluorescence microscopy, we demonstrate that WIPF2 is transiently localized to the site of bound conidia. Overall, we demonstrate the active role of the Arp2/3 complex and WIPF2 in mediating the internalization of *A. fumigatus* conidia into human airway epithelial cells. Also many study reported that Nystatin is the oldest polyene antifungal drug and exhibits a quite broad antifungal spectrum, turning it into a proficient candidate for the treatment of several fungal infections. It is active against almost all pathogens included in the "critical" and "high" aforementioned groups of the WHO list of priority pathogens (47). Nystatin presents both *in vivo* and *in vitro* activity against a broad spectrum of fungal pathogens such as *Aspergillus* (*A. niger, A. fumigatus, A. terreus,* and *A. flavus*), and also to several opportunistic pathogens of *Candida* species, like *C. albicans*, *C. auris, C. parapsilosis*, *C. kefyr, C. tropicalis*, *C. lusitaniae* and *C. dubliniensis* (48-50)

Wilson et al. (51) and Pappas et al. (52) mentioned that largely because of the increasing size of the population at risks, such as patients receiving immunosuppressive therapy, those undergoing bone marrow transplantation or other infection, tuberculosis or cystic fibrosis. In addition, the widespread implementation of fluconazole antifungal prophylaxis has rendered the host at greater risk for colonization with more resistant fungal species, enhancing the increase of invasive fungal infections in these already immune-suppressed patients. Additionally in Ashu et al. (53) study 195 isolates, 188 (96.4%) had the minimum inhibitory concentration (MIC) of AMB ≥2 mg/L, with approximately 80% and 20% of all clinical and environmental isolates having MICs of ≥ 4 mg/L. Overall, the clinical isolates were less susceptible to AMB than environmental isolates (*P*-value <0.001). The strain with the highest AMB MIC (16 mg/L) had one of the highest catalase activities. However, there was no correlation between AMB MIC and catalase activity. Concluded a widespread AMB resistance suggests that using AMB in the management of *A. fumigatus* infections in Hamilton would likely result in treatment failure. Although high catalase activity may have contributed to AMB resistance in some isolates, the mechanism(s) for the observed AMB resistance in Hamilton is unknown and likely complex..

However our outcomes did not agreed with Han et al. (54) as the nystatin protection assay was used to analyze the ability of *A. fumigatus* internalization. Experiments are performed in 3 independent experiments. The results indicated that complement receptor 3 (CD11b/CD18) is an important receptor that mediates adhesion, phagocytosis and chemotaxis in various immunocytes. The conidia of the medically-important pathogenic fungus, *Aspergillus fumigatus*

can be internalized into alveolar epithelial cells to disseminate its infection in immunocompromised host; however, the role of CR3 in this process is poorly understood. In the present study, we investigated the potential role of CR3 on *A. fumigatus* internalization into type II alveolar epithelial cells and its effect on host intracellular PA content induced by *A. fumigatus*. Results illustrated that CR3 is expressed in alveolar epithelial cells and that human serum and bronchoalveolar lavage fluid (BALF) could improve *A. fumigatus* conidial internalization into A549 type II alveolar epithelial cell line and mouse primary alveolar epithelial cells.

The aim of Takeda et al. (55) study was to determine the detection rate of azole-resistant *Aspergillus fumigatus* (ARAF) in isolates from chronic pulmonary aspergillosis (CPA) patients who were treated with azoles for varying durations *A. fumigatus* isolates (n = 120) were collected from CPA patients (n = 104), the isolates were tested for susceptibility to the azole drugs itraconazole (ITCZ) and voriconazole (VRCZ). The detection rate of ARAF among all isolates was 8.3% (n = 10). Of the 10 resistant isolates, eight were ITCZ-resistant and five were VRCZ-resistant. Among 47 isolates obtained from 36 CPA patients who were treated with ITCZ, ARAF was detected at a high rate in CPA patients, particularly in those with ongoing long-term azole treatment, at the time of azole antifungal therapy failure. One of the two main azole-resistance-acquiring mechanisms in A. fumigatus is a point mutation in *cyp51A* caused by long-term azole exposure among aspergillosis patients. The position of the mutation might determine the azole resistant phenotype, that is, single, multi, or panazole resistance (56).

Sani et al. (36) study was aimed to isolate, phenotypically identify, determine the prevalence of pulmonary fungal pathogens and antifungal susceptibility pattern of isolates of presumptive tuberculosis (PTB) patients, sputa were collected from 216 participants. Of the 216 participants, 19.9% had PTB and 73.6% had pulmonary fungal pathogens. Among the isolated pulmonary fungal pathogens, *Aspergillus fumigatus* made the highest occurrence, Penicillium citrinum, Mucor spp. and Aspergillus flavus are more susceptible to voriconazole, and Candida albicans was found to be more susceptible to Nystatin. Of the 159 fungal isolates, 92.5% were resistant to fluconazole.

5. Conclusion

The results showed an increase in the prevalence of *Aspergillus fumigatus* in Iraqi patients. This prevalence may be alarming on public health.

Compliance with ethical standard

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

All authors declare that informed consent was obtained from all individual participants included in the study.

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