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Original Article

Estimate correlation between Aspergillus Spp. and Asthma Patients

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Abstract:- Fungal exposure is a daily fact of human existence that infrequently results in disease; Asthma is a chronic inflammatory disease of the airways characterized by respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity. The relationship between day-to-day changes in asthma severity and combined exposures to community air pollutants and aeroallergens remains to be clearly defined. There is a close association between fungi and asthma, the so-called fungal asthma.

This study aimed to isolate the causative Aspergillus species that increase the risk of infection and to find out the association between asthma and Aspergillus spp. 40 sample of (sputum s and pharyngeal swabs) were collected from patients suffer from asthma symptoms. All samples were of both gender and of different age groups at the Specialized Center for Respiratory Diseases.

Samples were grown on two culture media (SDA and MEA) and incubated at 37°C for 7 days. The numbers of positive samples were 32 on SDA medium, 29 on MEA medium. Isolates were identified using special culture media characteristics as well as macroscopic and microscopic analysis. Where A. flavus was found to be the most predominant species of the sputum (58%) and from the pharyngeal swab (58.82%) on SDA, while on MEA the most prominent species were A. flavus and A. terreus (35%) of the sputum and the pharyngeal swab was A.niger (42.11%). The Aspergillus genera showed resistance to Fluconazole, Amphotericin B and Nystatin. The study has concluded that, there is a correlation between asthma and Aspergillus species. Aspergillus flavus the most common Aspergillus associated with lung diseases (Asthma).

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Introduction:

Asthma is a chronic airway inflammatory disorder associated with airway hyper responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness and coughing particularly at night or early morning (Bruce et al., 2012). Asthma can be classified into phenotypes and endotypes based on clinical symptoms, histology, genetics, therapy response, and other factors (Campo et al., 2013). One of several elements that influence asthma morbidity is the environment (Gold et al., 2017). Temperature, allergies, smoking, dust, mites, and other factors can all contribute to an asthma attack (Murray and O'Neill, 2018). Intermittent, mild, moderate, and severe asthma are the four levels of severity (Nunes et al., 2017).

Allergic diseases are multifactorial disorders in which genetic, environmental, and socioeconomic factors interact to determine disease manifestation and result in various phenotypes. Smoking, heredity, biomass usage, occupational risks, environmental smoke exposure, lifestyle, poor nutrition, and poorer socioeconomic status are the key risk factors for all types of respiratory diseases (Foo et al., 2016; Feshchenko et al., 2017).

Fungi are eukaryotic, non-chlorophyllous and multicellular (molds) or unicellular (yeast) organisms that are mostly spore-bearing and inhabit different environmental sources such as soil, plant parts (leaves, roots, and fruits), water, and food sources (Maheswari and Komalavalli, 2013). The growth and distribution of fungi are affected by different environmental factors such as temperature, pH, moisture, degree of aeration, amount and type of nutrients (Gaddeyya et al., 2012).

1.2 Aims of Study

General Objective:

To know the relationship between Aspergillus spp. and Asthma.

Main Objective:

1- Isolate the Aspergillus spp. causes associated with asthma that increase bronchial sensitivity in asthmatic patients and identify the dominant species.

2 Literatures Review

2.1 Asthma

2.1.1 Definition of Asthma

Asthma is considered to be a chronic inflammatory disease that may be resulted from the interaction of environmental and genetic factors (Mims, 2015). Most clinical features of asthma are represented by airway obstruction, bronchial hyper responsiveness, and greater airway wall thickening, with episodes of various symptoms, including chest tightness, wheeze, shortness of breath, and dry cough, particularly at night or in the early morning (Mims, 2015). The prevalence of asthma follows a characteristic age and gender related pattern, being highest during childhood and predominantly affecting males (Gerritsen, 2008). In adults, the prevalence of asthma in females is higher than in males, while before puberty in boys it is higher than in girls (Postman, 2007).

Thus, in order to manage asthma in females, the relationship between hormonal variation and asthma symptoms should be focused on (Noori Ali and Abdul Hussein, 2019).

2.1.2 Causes of asthma

Asthma is caused by a combination of complex and incompletely understood environmental and genetic interactions (Ishiguro et al., 2014). These affect the condition's severity as well as how well it responds to therapy (Chakrabarti and Slavin, 2011). It is believed that the recent increased rates of asthma are due to changing epigenetics (heritable factors other than those related to the DNA sequence) and a changing living environment (Jan et al., 2008). Asthma that starts before the age of 12 years old is more likely due to genetic influence, while onset after age 12 is more likely due to environmental influence (Moreto et al.,2011).

2.1.3 Signs and Symptoms

The symptoms of asthma include coughing, chest tightness, sputum production, and dyspnea (shortness of breath), as

well as chronic inflammation that is associated with variable airflow obstruction, bronchial hyper responsiveness, recurrent episodes of wheezing that may occur a few times a day or a few times per week, and bronchospasm (Hirose et al., 2013). The symptoms are usually worse at night and in the early morning or in response to exercise or cold air (Hirose et al., 2013).

The prevalence of asthma symptoms varies among individuals. Some suffer from mild symptoms only, while others have severe symptoms, and the attacks may occur suddenly or after relatively symptom-free periods. An acute asthma attack can be life-threatening if treatment is not sought immediately (Rogers, 2004).

2.1.4 Etiology and Risk factors for asthma

Asthma includes considerable genetic and environmental components that interact in the disease's development and subsequent manifestation (Holgate, 2011). Infections and endotoxin exposure, for example, may be protective or risky, depending on the timing of exposure in infancy and childhood (Subbarao et al., 2009).

Table 2.1: Factors affecting the development of asthma (Busse *et al*., 2001)

Host Factors	**Factor Type**
Genetic	•Genes pre-disposing to atopic. •Genes pre-disposing to airway hyper responsiveness
Obesity	
Sex, Age, Race.	
Environmental Factors	
Allergens	• Indoor: Domestic mites, furred animals (dogs, cats, mice), cockroach allergen, fungi, molds, yeasts • Outdoor: Pollens, fungi, molds, yeasts (predominantly viral)
Infections	Respiratory infections (virus and fungi)
Occupational sensitizers	
Tobacco smoke	• Passive smoking • Active smoking
Outdoor/Indoor Air Pollution	

2.2 Fungi

Fungi are one of the kingdoms of life; they are eukaryotic, non- chlorophyllous organisms dependent on external sources for nutrition. They exist as saprophytes, symbionts, or parasites on animals and plants, ubiquitously in all environmental niches, including the human body. These ubiquitous organisms, which grow in intra and extra-domiciliary environments and on almost any substrate, exhibit optimal growth at a temperature of 25-30 °C (Simon-Nobbe et al., 2008). It can be broadly categorized into mushrooms, yeasts, and filamentous fungi (molds) (Hawksworth, 2001). The currently accepted classification of fungi includes one subkingdom, seven phyla, ten subphyla, 35 classes, 12 subclasses, and 129 orders (Hibbett et al., 2007).

2.3 Aspergillus ssp.

Filamentous Aspergillus is a commonplace fungus that usually infects immunocompromised hosts and people with underlying lung conditions (Latgé and Chamilos,2019). Aspergillus species reproduce asexually by conidia and get their nourishment from decomposing matter in the environment (Henß et al., 2022; Latgé and Chamilos,2019). Over twenty-four species of Aspergillus are capable of causing human disease, but A. fumigatus, followed by A. terreus and A. flavus, is the most implicated as a pathogen (Woodring et al.,2023).

Aspergillosis is caused by fungus of the same genus, but it should be understood as a spectrum of processes that vary greatly according on the immunological condition of the host (Castro-Fuentes et al., 2022). Despite the fact that

conidia are spread by aspiration, most inhalers of conidia will not develop aspergillosis because of their immune system's defenses (Montone, 2016). The most significant immune cell in the immunological response against Aspergillus species is thought to be neutrophils (Daly and Kavanagh, 2001).

2.3.1 Relationship between Aspergillus and asthma

The relationship between fungus and asthma, especially Aspergillus fumigatus, has been the subject of extensive research. The ubiquitous presence of A. fumigatus, its thermotolerant nature, the repairable size of its conidia, and its ability to produce potent allergens are pivotal in worsening asthma control. Due to the diverse clinical manifestations of fungal asthma and the lack of specific biomarkers, its diagnosis remains intricate (Agarwal et al.,2023). Depending on the severity, patients with fungal asthma require personalized treatment plans, including inhaled corticosteroids and bronchodilators, and antifungal therapy (Agarwal et al.,2023).

2.4 Fungal Identification

Early diagnosis of fungal infection is critical to effective treatment. There are many impediments to diagnosis, such as a diminishing number of clinical mycologists, cost, time to result, and requirements for sensitivity and specificity (Kozel and Wickes, 2014). Diag++nosis of fungal infection has relied primarily on methods such as direct microscopic examination of clinical samples, histopathology, and culture. Such approaches are dependent on personnel with relatively high levels of specific mycology training. As a consequence, there is an increased emphasis on the use of molecular methods and antigen detection as surrogates for culture in the diagnosis of fungal diseases (Kozel and Wickes, 2014).

3 Materials and Methods

3.1 Materials

The apparatuses and instruments were used in this study.

3.1.1 General Apparatuses and Instruments:

The main equipment and machines, their companies and origins were usedain the study are listed in (Table 3.1)..

Table (3.1): Shows machinery, equipment and their companies used in this study

NO.	Name of the device	Companies	Origin
1	Autoclave	Hirayama	Japan
2	Compound light microscope	Feica	Germany
3	Electronic balance	Denver	Germany
4	Incubator	Fisher	England
5	Oven	Memmert	Germany
6	Hood-cabinet	APDO	Jordan
7	Distillatory	Buchi	Switzerland
8	Refrigerator	Hitachi	USA
9	Burner	Amal	Turkey
10	Hot plat with Magnetic Stirrer	Gallenkamp	UK

3.1.2 General Tools

The main tools, their manufacturing companies, and their origins used in the study are listed in (Table 3.2).

Table (3.2): Shows the tools, facilities, and companies used in this study

NO.	Name of the tool	Companies	Origin
1	Ice box	Tank	Egypt
2	Disposable petri dishes	AL-Hani	Lebanon
3	Disposable- loop	China	China
4	Flask	Chemical-Lab	China
5	Cotton Swab	AL-Hani	Lebanon
6	Graduated cylinders 1000ml	Chemical-Lab	China
7	Transport Cotton Swab with media	Promega	USA
8	Gloves	Berika	Turkey
9	Slide	Promega	USA
10	Cover slide	Promega	USA
11	Loop	Gesalife	Germany
12	Racks for Eppendorf tubes	Meheco	China
13	Eppendorf tube (2ml)	Promega	USA

3.1.3 Biological and Chemical Materials

The materials used in this study are listed in (Table 3.3).

Table (3.3): Different biological and chemical materials

NO.	Materials	Company	Origin
1	Agar-agar	Hi-media	India
2	Absolute ethanol	Fluka	Germany
4	Chloramphenicol	Samara	Iraq
7	Deionized water	Bioneer	Korea
8	Lacto-phenol cotton blue dye	Hi-media	India

3.2 Methods

3.2.1 Subjects

3.2.1.1 Patients Study Group

This group consists of 40 patients previously diagnosed with asthma. Their ages ranged from (10 to 58) years, including (16males and 24females), who underwent the Specialized Center for Respiratory Diseases.

3.2.1.2 Samples collection and preparation

40 sputum and pharyngeal swab samples were collected from asthma patients from different places.

3.2.2 Sterilization Methods

3.2.2.1 Wet-heat sterilization

The buffer, solution, and culture medium were sterilized using this method by utilizing an autoclave at 121 °C with a pressure of 15 lbs/in² for 15 minutes.

3.2.4 Isolation and Identification of the Aspergillus Isolates

Filamentous fungi species (Aspergillus spp.) were isolated from sputum and pharyngeal swab and identified by relying on phenotypic characteristics and microscopically as reported in taxonomic sources, which included:

3.2.4.1 Morphology Examination of Isolated Fungi

The fungal (Aspergillus spp.) morphology was studied macroscopically by observing the colony features (color, shape, texture, size, and hyphae), the growth rate, and size of colonies on standardized media (Tiwari et al., 2011). If a culture contained more than one type of spore or colony morphology, isolates were subcultured on SDA and MEA until the characteristics were consistent.

3.2.4.2 Microscopically Examination of Isolated Fungi

The micro-features of pure culture were determined by semi-permanent microscopic preparations of fungi using lacto-phenol cotton blue stain (Chaverri et al., 2015). A drop of lacto-phenol cotton blue stain is placed on a clean slide before transferring a small tuft of fungus with a sterilized inoculating loop. Then the slides are gently heated to release air bubbles and observe under compound microscope for the conidia, conidiophores and arrangement of spores with a small portion of the mycelium (Gaddeyya et al., 2012).

3.3 Statistical analysis

The effects of media, age and gender on frequency isolations were examined using the SPSS software (IBM SPSS statistics for windows, Version 25, IBM Corp, Armonk, NY, USA). One Test used (Chi square). The results of the statistical analysis were reported as statistical significance level used was $P \leq 0.05$ for all factors.

4 Results

4.1 Identification Culture samples of asthmatics

In this study, 40 asthmatic samples of sputum and pharyngeal swabs were cultured on two culture media (SDA and MEA). The results showed that the number of positive samples was higher than negative samples. Statistically, there is significant difference ($P \leq 0.005$) between the positive and negative samples of the patient groups on SDA and MEA, as shown in (Table 4.1).

Table (4.1): The percentage of positive and negative culture media samples in asthmatic patients

Media	Positive Samples		Negative Samples		Total	p-value	OR
	NO. Samples	Percentage	NO. Samples	Percentage			
SDA	32	80 %	8	20 %	40	0.009	0.94
MEA	29	72.5 %	11	27.5 %	40	0.011	0.82

4.2 Isolation and Identification of the Aspergillus Isolates

4.2.1 Macroscopic and Microscopic Features of Isolated Aspergillus spp.

In this study, the isolations Aspergillus were examined on the basis of cultural, microscopic, and morphological characteristics. The identification of Aspergillus spp. is based on the macroscopic characteristics of colonies grown on culture media (SDA and MEA). The colony's size, color, and form are all essential features for species identification. As shown in (Figure 1). Aspergillus spp. diagnosed by morphological examination was A. niger, A. flavus and A. terreus.

The microscopic features in lactophenol cotton blue (LCB) stained wet mounts were used to identify Aspergillus spp. The visualization of conidiophores

4.2.2 Aspergillus isolation frequency

4.2.2.1 Effect of media on the frequency of Aspergillus isolation

In general, the identification of Aspergillus is based on morphological and microscopic diagnosis. The frequency of sputum sample isolation showed that in the SDA the community consisted mostly of Aspergillus flavus (58%),

followed by Aspergillus niger (42%). As for the pharyngeal swab that showed the most presence of Aspergillus. flavus (58.82%) and A.niger (41.18%).

For MEA, the predominant Aspergillus genera/species from sputum sample were A. flavus (35%) and A. terreus (35%), followed by A. niger (30%). As for the pharyngeal swab that showed the most presence the A. niger (42.11%), followed by, A. terreus (31.57%) and A. flavus (26.32%).

The best media for isolation were SDA and MEA, respectively (Figure 4.4). Statistically, (Tables 4.2) showed that the effect of media on the frequency of isolation of Aspergillus spp. isolated from patients suffering from asthma symptoms.

Table (4.2): the effectiveness of media on *Aspergillus* isolates percentage isolated from asthmatic patients

Sample Type	Media (% of isolates)		Total
	SDA	MEA	
Sputum	24 (58.54 %)	20 (51.28 %)	44 (%) 54.91
Pharyngeal Swab	17 (41.46%)	19 (48.72%)	36 (45.09%)
Total	41 (51.25%)	39 (48.75%)	
p-value	0.07	0.03	80 (50%)
OR	0.043	0.039	

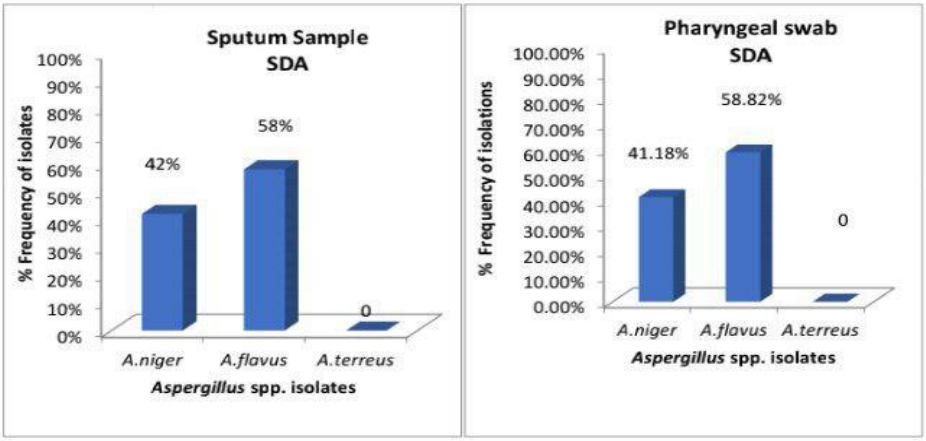

Figure 4.2 (a, b): The frequency of *Aspergillus* species isolated from sputum and pharyngeal swabs on Sabouraud Dextrose Agar medium at 37°C for 7 days.

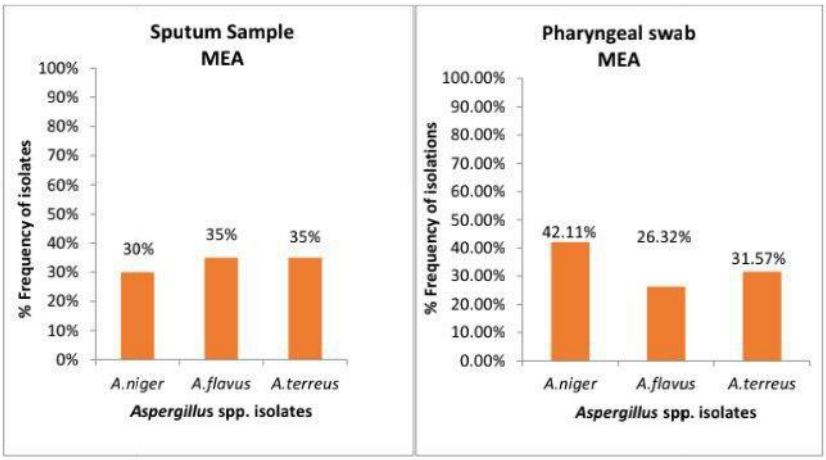

Figure 4.3(a, b): The frequency of *Aspergillus* species isolated from sputum and pharyngeal swabs on Malt Extract Agar medium at 37°C for 7 days.

4.2.2.2 Effect of gender on frequency of Aspergillus isolation

The results found that females (52.5%) had a higher frequency of isolation than males (47.5%), and there was a clear effect of sex on the frequency of isolation of Aspergillus species when cultured sputum and pharyngeal swabs samples. The statistical results for the efficacy of sex on the frequency of isolation of Aspergillus species examined are shown in (Tables 4.3).

Table (4.3): Statistical analysis Effect of the gender of on frequency isolations by using Chi –square test

Gender	No. of samples	No. of isolates (%)	p-value
Male	20	38 (47.5 %)	0.00 S
Female	20	42 (52.5 %)	
Total	40	80 (100%)	

4.3 Antifungal resistance

The current study included the use of four antifungals to test three genera of Aspergillus. The results showed that all Aspergillus spp. were sensitive to Ketoconazole. On the other hand, A. niger and A. terreus were sensitive to Fluconazole, while A. flavus was resistance against Fluconazole. Also, the findings reported that A. flavus and A. niger were sensitive to Nystatin, while A. terreus was resistance, but all species except A. niger were sensitive to Amphotericin B. Theost effective antifungal against Aspergillus spp. was Ketoconazole, (Table 4.5).

Table 4.5: Summarize the effectiveness of different antifungals required to inhibit *Aspergillus* growth by three *Aspergillus* spp on SDA medium at 37°C.

Aspergillus spp.	Resistance/ Sensitivity (mm)			
	Azoles		Polyene	
	Fluconazole	Ketoconazole	Nystatin	Amphotericin B
A. flavus	R	+	+	+
A. niger	+	+	+	R
A. terreus	+	++	R	+

Key: R = Resistance; ++ = Sensitive (10 mm); + = (4 mm)

Discussion

5.1 Identification Culture samples of asthmatics

This study is a case-sectional study. The range of positive samples in asthma patients was higher. There were also clear differences between positive and negative samples of asthma patients. The results of the study showed that the number of positive samples of patients groups (32 SDA, 29MEA) is higher than the negative, and this leads to the fact that fungi have a role in asthma.

A number of subjects had more than one Aspergillus isolated from their sputum and pharyngeal swabs, suggesting either heavy exposure or, perhaps more likely, a defect in host defense against fungi, making them susceptible to colonization. Such defense involves a combination of innate and adaptive immunity, and the extent to which there is a deficiency in any of these pathways in some people with asthma is unknown (Dumestre-Pérard et al., 2008).

5.2 Isolation and Identification of the Aspergillus Isolates

5.2.1 Macroscopic and Microscopic Features of Isolated Aspergillus spp.

In this study, the Aspergillus isolates were first identified at a genus level using a morphological examination depending on the colors of colonies formed on both sides of petri dishes, the top and reverse of the fungal cultures. The microscopic examination of the shape of the spore-producing structures was used for further identification. The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level (Wang et al., 2016).

The results of the present study revealed that A.flavus had a higher rate compared to Aspergillus species (46.5%) in sputum samples, while A.terreus had the lowest rate (17.5%). These results have agreed with (Salman and Al-haddad, 2021), where they found that A. niger (10.81%) came second. The result of the current study in disagreement with (Denis et al.,2018), mentioned that the more common fungal was A. fumigatus (57.5%).

5.2.2 Aspergillus isolation frequency

5.2.2.1 Effect of media on the frequency of Aspergillus isolation.

The Aspergillus genera were isolated using two types of culture media: SDA, and MEA. The highest number of fungi was obtained from SDA (80%), followed by MEA (72.5%). The current study showed that the media has an effect on fungal isolates through the different numbers of isolates of each of the medium and the different numbers of some Aspergillus species produced by the same medium. This study agreed with the study (Pashley et al., 2012), that the choice of media on the tested media has an effect on the detection of fungi from respiratory samples.

5.3 Antifungal Resistance

Antifungal susceptibility testing methods are available to detect antifungal resistance and to determine the best treatment for a specific fungus. Clinical microbiology relies on these methods to select the agent of choice for a fungal infection, and to know the local and global epidemiology of antifungal resistance (Alastruey-Izquierdo et al., 2015).

In our study, in-vitro susceptibility for Aspergillus species was performed using the disc diffusion method and found that the most effective antifungals were Ketoconazole, while some species the resistance was most commonly seen against Nystatin, Amphotericin B and Fluconazole. These results agree with those of (Khan et al., 2015), where it was found that Amphotericin B and Fluconazole is resistant and with a study (Rudramurthy et al.,2013), it was found that Amphotericin B is sensitive to A.flavus. The results agreement with Kumar et al., (2010), who found that the A. flavus isolates were sensitive for Amphotericin B and Ketoconazole except fluconazole which showed resistance

Conclusions

1-There is a relationship between exposure to fungi (Aspergillus spp) and asthma.

2- A. flavus is the most common species of Aspergillus that appeared during this study.

3- Ketoconazole is the most effective antifungal against asthma caused by Aspergillus fungus.

Recommendations

1-Stay away from allergy irritants and smoking as much as possible because they cause an increase in the severity and exacerbation of asthma

2-Increasing studies on fungi that cause diseases in the respiratory system because they produce mycotoxins in the human body and thus pose a threat to their health.

3-Reliance on other modern confirmatory diagnostic methods (non- conventional) because the give more accurate results.

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