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Evaluation of Some Aspergillus Mycotoxins in Patients with Bronchitis in Al-Najaf City

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ABSTRACT: This research aimed to establish whether the Aspergillus species identified from bronchitis patients were mycotoxigenic or non-mycotoxigenic fungi in sputum and serum samples. A total sample of 170 subjects with ages ranging from 15 to 72 years— were obtained from Al-Najaf Province (Al-Sadr Medical City) between December 2023 and April 2024, divided into patient (n=176) and healthy groups (n=34). The Aspergillus genera isolated were cultured on coconut agar medium and screened under UV light (360 nm); each sputum and serum sample was also tested using the ELISA technique. All Aspergillus strains exhibited blue fluorescence on Coconut Cream Agar (CCA) media under UV light, confirming positivity. The study identified production of aflatoxins B1 and ochratoxins-A in sputum and serum samples among both patients with bronchitis and control groups. The results showed a significant difference in patient groups by samples ($P \leq 0.05$) while the healthy groups showed a non-significant ($P>0.05$) difference. Most Aspergillus spp. Isolated from bronchitis patients were found to produce aflatoxin B1 and ochratoxin-A on coconut agar medium— mycotoxins extracts included sputum (about 68.4% for aflatoxins-B1 and 41.7% for serum samples) and sputum (about 77.6% for ochratoxin-A and 91.7% for serum samples) taken from bronchitis patients, which were identified using ELISA technique.

KEYWORDS: Bronchitis, Mycotoxin, Aflatoxin, Ochratoxin, Aspergillus.

INTRODUCTION

Mycotoxins are not primary metabolites but secondary ones— they're toxic and produced by filamentous fungi that naturally contaminate agricultural commodities all over the world. A number of different species from Claviceps, Fusarium, Penicillium, and Aspergillus genera produce various types of mycotoxins among which ergot alkaloids, trichothecenes, zearalenone, fumonisins, patulin, ochratoxin A, and aflatoxins are the most important for food safety due to their immunosuppressant estrogenic nephrotoxic neurotoxic cytotoxic teratogenic mutagenic carcinogenic properties [1]

Over 300 mycotoxins are currently identified, originating from a diverse range of fungi with wide variations in structure and function, despite only few having significant biological effects on health. Examples include Aflatoxins (Afs), Ochratoxins (OTA), Fumonisins (FBs), Deoxynivalenol (DON), Patulin, Zearalenone and the various types of Trichothecenes [6].

Aflatoxins are produced as secondary metabolites by Aspergillus flavus and Aspergillus parasiticus molds, and their impact is predominantly concerning due to the adverse effects they have on human and animal health— including being carcinogenic, mutagenic, teratogenic, and immunosuppressive [7]. The biosynthesis pathway of aflatoxin is greatly influenced by various abiotic factors like temperature, water activity, pH, carbon and nitrogen; however, the synthesis itself is primarily dependent on temperature and water activity levels. All known types of aflatoxins can be classified into 18 different groups based on chromatographic and fluorescence characteristics [8]. These include AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2 among others with AFB1 being the most significant type as it is classified as a class 1 carcinogen by IACR [9]. Aflatoxicosis is mainly associated with Aflatoxin B1 — responsible for acute toxicity — apart from chronic toxicity leading onto carcinogenicity plus teratogenicity as well as genotoxicity via immunotoxicity [10].

Ochratoxins are mycotoxins: a group of secondary metabolites produced by storage fungi, primarily Penicillium and Aspergillus species, with ochratoxin A (OTA) standing as the most prevalent [11]. The production of OTA is contingent upon various factors including temperature, water activity (aw), and specific low-consumed elements [12]. The actualization of ochratoxin A is reliant on fungous resources— influenced by product type and geographical whereabouts. OTA exhibits carcinogenic nephrotoxic teratogenic immunotoxic neurotoxic properties — having been implicated in nephropathy among

humans[13]. This study concerns to aflatoxins B1 plus ochratoxins-A appearance in sputum and serum samples obtained from bronchitis patients.

MATERIAL AND METHODS

During the period from December 10th, 2023 to May 25th, 2024, this cross-sectional case-control investigation was carried out in Al-Najaf Province (Al-Sadr Medical City). A total of 170 individuals of both sexes aged between 15 and 72 years were included. The participants were split into two groups: group A comprised 136 bronchitis patients, while group B consisted of 34 healthy control subjects.

DETECTION TESTS

Coconut-based medium test

A Coconut Cream Agar (CCA) was concocted as per the prescribed instructions from the manufacturer: immersing 50g of coconut cream and 2g of agar in 100ml of sterile D.W. The media was heated in a beaker on a heating centrifuge till it reached boiling point— ensuring complete dissolution of the medium. Sterilization was carried out for 15 minutes in an autoclave at 15 pounds pressure and 121 degrees Celsius; allowed to cool to about 45-50°C before pouring into sterile Petri dishes. Following this, a sample suspension was prepared by introducing 1ml of sterile normal saline into an eppendorof tube, then vortex-mixing part of the colony taken using a sterile loop with the saline. The plate center was then inoculated with 5 µl of spore suspension and placed in incubation, shielded from light at a constant temperature of 25°C. Agar was inspected under UV light after colonies were allowed to grow for a period of 3-7 days. The presence or absence of a fluorescence ring around the colonies was observed, and based on this observation, results were recorded either as positive or negative.

ELISA Technique

This used a kit provided for Enzyme-Linked Immunosorbent Assay (ELISA). The plate comes precoated with the human (OCH-A; AKR7L) antibody: when (OCH-A; AKR7L) from your sample is added, it binds to the antibodies on the well. Subsequently, biotinylated human (OCH-A; AKR7L) antibody is introduced— binding to the (OCH-A; AKR7L) already present in your sample. This is followed by the addition of Streptavidin-HRP that binds to the biotinylated (OCH-A; AKR7L) antibody after incubation: any unbound Streptavidin-HRP is removed through a washing step. The color that you observe upon adding substrate solution indicates the amount of Human (OCH-A; AKR7L) present, as it develops proportionally. Terminate this reaction with an acidic stop solution and use a spectrophotometer to measure absorbance at 450 nm.

Statistical Analyses

The statistical program SPSS-25 was used to analyze the results. Simple frequencies and percentages were employed as the presentation format for the data. The significance of the difference between distinct percentages (qualitative data) was determined using the Pearson Chi-square test. Statistical significance was considered when the P-value fell below 0.05. Chi square analysis was performed to test the relationship between categorical variables..

RESULTS

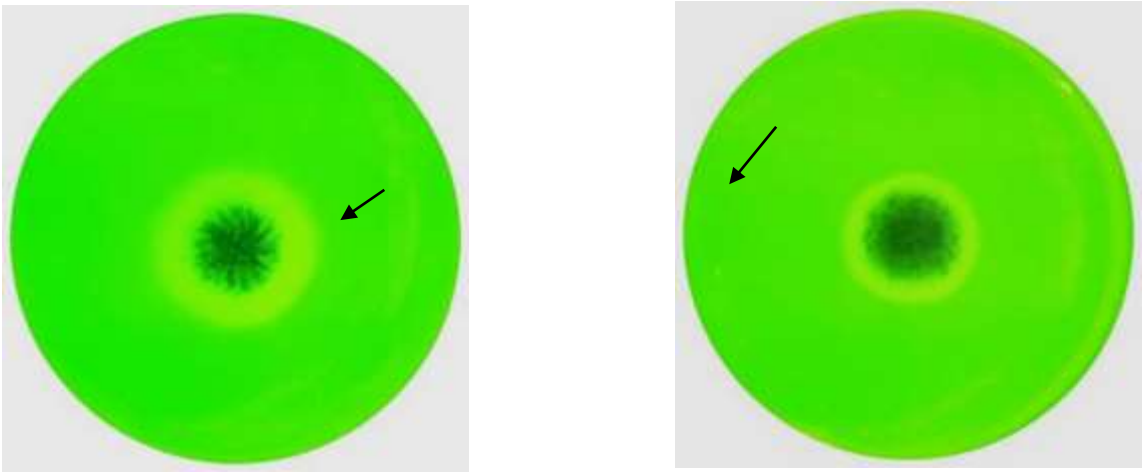
Screening for mycotoxin production by *Aspergillus* on Coconut Cream Agar

Five Aspergillus species were studied— A. flavus, A. parasiticus, A. nomius, A. wenti, and A. ochraceus— all isolated from sputum samples to determine their mycotoxin production capability using the fluorescence technique on CCA medium (refer to Figure 1). All tested strains of Aspergillus demonstrated positive results indicated by blue fluorescence when exposed to UV light on CCA media (table 1).

Table 1. Qualitative evaluation of the ability of different Aspergillus species to produce mycotoxins on a coconut cream agar medium (CCA).

Strain	Mycotoxins production
A. flavus	+++
A. parasiticus	++
A. nomius	+
A. ochraceus	+
A. wenti	+

Key of test: (+) Traces of production, (++) Moderate production, (+++) Strong production



(+++) **(+)**

Figure 1: The reverse of CCA cultures of *Aspergillus* isolated from bronchitis patients. The positive strains fluoresce green

AFLATOXINS B_1 ANALYSIS

Table (2) demonstrates the aflatoxin B_1 detection percenatsge in patients with bronchitis sputum and serum. According to this table, the sputum appears to be the best specimen to test when looking for aflatoxins. Bronchitis patients showed positive results in (68.4%) of sputum samples, while only (44.4%) of healthy individuals tested positive. Interestingly, it was noted that serum is not a reliable reservoir for detecting aflatoxin levels. The reported levels of mycotoxins found in sputum samples were between 0.11-1.766 ppb or ng/mL, whereas for serum samples it was much lower at 0.01-0.499 ng/ml— despite higher levels being detected among patient groups compared to healthy ones. Statistically, the patient groups showed a significant difference in levels by samples ($P \leq 0.05$) and the healthy groups showed a non-significant ($P > 0.05$).

Table 2. Distribution of Aflatoxin B_1 in the sputum and serum samples among patients and healthy groups

Aflatoxin B_1	**Patients n = 136**		
	Sputum (n=76)	**Serum** (n=60)	***Chi square (P vaue)***
Positive	52 (68.4%)	25 (41.7%)	9.77 (0.002) HS
Negative	24 (31.6%)	35 (58.3%)	
	Control n = 34		
	Sputum (n=27)	**Serum** (n=7)	***Chi square (P vaue)***
Positive (n=27)	12 (44.4%)	2 (28.6%)	0.58 (0.44) NS
Negative (n=7)	15 (55.6%)	5 (71.4%)	

HS: High Significant at P < 0.01 ; NS: Non-Significant at P >0.05

b) Ochratoxins-A Examination

Table 3 exihibits the Ochratoxin-A levels in patients with bronchitis' sputum and serum. As can be seen from Table, the sputum appears to be the best specimen to test when looking for ochratoxin-A. The percentages of positive sputum samples for bronchitis patients were 77.6%, while for healthy individuals it was 44.44%. On the other hand, the percentages of positive serum samples for bronchitis patients were 91.7%, with no positive samples from healthy individuals. The mycotoxin detection levels in sputum samples were found to be in the range of (1–51) ng/ml, while those in serum samples were in the range of (1–15) ng/ml— indicating higher levels in patient groups compared to healthy groups. Statistically, the patient groups showed a significant difference in levels by samples ($P \leq 0.05$) and the healthy groups showed a non-significant ($P > 0.05$).

Table 3. Distribution of Ochratoxin –A in the sputum and serum samples among patients and healthy groups

Ochratoxin –A	**Patients n = 136**		
	Sputum (n=76)	**Serum** (n=60)	***Chi square (P vaue)***
Positive	59 (77.6%)	55 (91.7%)	4.87 (0.02) S
Negative	17 (22.4%)	5 (8.3%)	
	Control n = 34		
	Sputum (n=27)	**Serum** (n=7)	***Chi square (P vaue)***
Positive	2 (7.4%)	0 (0%)	0.55 (0.45) NS
Negative	25 (92.6%)	7 (100%)	

S: Significant at P <0.05 ; NS: Non-Significant at P >0.05

DISCUSSION

From clinical aspects, respiratory aspergillosis has various forms, which include invasive pulmonary and extrapulmonary aspergillosis, extra-pulmonary colonizing, saprophytic pulmonary, extrinsic allergic alveolitis, allergic bronchopulmonary aspergillosis, and extrinsic asthma. ABPA develops in 1–15% of individuals globally already harboring cystic fibrosis, and also in 2.5% of asthma patients. The combined total equates to 4.8 million people worldwide [14].

The ineffectiveness of using coconut media for aflatoxin detection due to Aspergillus's high sensitivity is noted. A study cited found that coconut cream agar (50% coconut cream) was more effective than dried coconut medium. Results from the current study show that A. flavus and A. parasiticus produce mycotoxins at high levels while other species show varied levels of production [15], contradicting findings by Yazdani et al. where negative results were recorded for all isolates under UV; no fluorescent rings appeared [16].

The current study has found that mycotoxins were detected in the sputum and serum of normal healthy individuals, this result comes in agreement with what was previously mentioned in literature [17]; According to Kosmidis and Denning (2015), invasive aspergillosis has been reported to occur even in immunocompetent hosts when exposed to large quantities of aspergillus spores. [18]. Aydin and colleagues conducted a research in 2014. Their aim was to analyze the serum levels of four different types of aflatoxins (aflatoxin B1, aflatoxin B2, aflatoxin G1 and aflatoxin G2) among healthy adults. Their findings revealed that the average concentration found in the total population for AF was 1·33 ppb with a range varying from 0·15 to 3·38 ppb. [19]. On the other hand, Hmaissia Khlifa performed an investigation for the purpose of establishing ochratoxin A in human serum samples obtained from Tunisia by employing high performance liquid chromatography (HPLC) with fluorescence detection. It was observed that the average value of ochratoxin A for the healthy group was 0.49+/-0.79 ng/mL, as highlighted in the report by Khlifa. [20].

This study has used ELISA to detect mycotoxins in serum samples. A wide range of commercial ELISA-based kits are available for different matrices, including human serum, which makes it a highly versatile methodology. However, the potential for cross-reactivity with metabolites of target compounds or matrix components can give rise to overestimated values [21]. For this reason, AOAC International has not approved any ELISA method [22], and positive results must be confirmed (e.g., through chromatographic methods).

No research has ever demonstrated large quantities of various body fluids to be contaminated with mycotoxins. But this study points out that mycotoxins are present in body fluids (sputum and serum) of humans post environmental exposure to toxin producing molds— which could play a role in bronchitis pathogenesis. It was discovered that Aflatoxins — secreted by respiratory A. flavus — can impair motile as well as chemosensory functions of airway cilia; thus contributing towards the development of fungal airway diseases. This information is helpful to understand the situation when it comes to consideration for health impacts resulting from mycotoxin contamination in the environment [23].

The research revealed that both the patient and control groups exhibited higher levels of aflatoxinsB1 and ochratoxins-A in positive samples compared to negative samples in sputum samples. Moreover, it indicated that mycotoxins were more abundant in patient samples than in control samples. Interestingly, divergent results were observed in serum samples; while the negative samples showed higher presence of aflatoxin B1 for patients and controls, high levels of ochratoxin-A positive samples were

detected for both patients and controls. These findings stand in contrast with those reported by Hooper et al., they identified higher quantities of aflatoxinB1 and ochratoxins-A in positive sputum samples, which differs from our results significantly [24].

CONCLUSION

The results of the research indicated that the majority of Aspergillus spp. found in the bronchial samples of bronchitis patients were mycotoxigenic molds, which could be identified by their production of mycotoxins on coconut cream agar medium (CCA) upon UV light screening. Aflatoxin B1 and Ochratoxin-A were discovered in both sputum and serum samples; interestingly, these mycotoxins were more prominently present in sputum rather than serum samples.

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