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Virulence Potential of *Aspergillus flavus* Isolated from Dogs

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Abstract | *Aspergillus flavus* is the cause of invasive and non-invasive Aspergillosis. The secretion of hydrolytic enzymes is considered a virulence factor in this species. The question of whether *Aspergillus* species can actually create biofilms has gained attention in recent times. Our work aimed to isolation and identification of *Aspergillus flavus* from dogs with study and evaluate some of virulence factors of this fungus like proteinase, hemolytic activity and biofilm formation in Laboratory. Fifty clinical isolates of respiratory infections in dogs were collected from several locations in Baghdad from Al Rusafa district (Zayouna, Al-Mashtel Street, Adamiyah; and Al-Karkh district (Baghdad Veterinary Hospital) during November 2023 to January 2024. The macroscopically and microscopically examination of this fungus was used to diagnosis of fungal elements. After that, detection of virulence factors for *Aspergillus flavus* was done using special media for hydrolysis albumin, hemolysis action and microplate technique for biofilm formation. This study showed a high percentage of *Aspergillus* spp.; at 42%, which included (*Aspergillus flavus*, *Aspergillus niger* at 18% respectively and *Aspergillus terreus* at 6%), The second fungus which isolated was *penicillium* spp. at 24% which also appeared an opportunistic filamentous fungal infections in dogs, with other fungi isolated at 34%. All isolates of *Aspergillus flavus* investigated highly significant differences in the hydrolysis of proteinase, hemolysis, and biofilm formation. *Aspergillus flavus*'s biofilm development showed the highest degree of virulence; these findings suggest that the biofilm phenotype is a more significant concept in mycological research.

Keywords | *Aspergillus flavus*, Dogs, Proteinase, Biofilm, Hemolytic activity

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INTRODUCTION

The most opportunistic pathogen is *Aspergillus flavus*, which infect humans and other animal species both superficially and invasively (Steinbach *et al.*, 2012; Khodavaisy *et al.*, 2016). In order to infect the host, hazardous virulence factors are required for *Aspergillus* strains. *Aspergillus* species produce virulence factors that facilitate the development of an infection and may subsequently lead to invasive or widespread infections. In recent years, the significance of *Aspergillus* infections has increased (Sales *et al.*, 2013). The most typical form

of *Aspergillosis* in dogs is a localized infection of the nasal region. In dogs systemic Aspergillosis usually affects the thoracic lymph nodes, lung, and renal pelvis (Tell, 2005). The production of a broad spectrum of hydrolytic enzymes, adhesions, heat resistance, pigment synthesis, and toxic metabolites are thought to be additional virulence characteristics to support *Aspergillus* species pathogenesis and enable the fungal cells to evade the host's defense (Singh and Urhekar, 2017). Aspergillosis pathogenesis is determined by host variables (Immune state) as well as pathogen virulence factors. Extracellular hydrolases are among the principal virulence factors, suggesting that

they play an important role in *Aspergillus* spp. widespread colonization of lung tissue. Enzymes such as α -amylase, proteinase, phospholipase, pectinase, and lipase play a significant role in degrading tissue and erythrocytes lyse (Vila and Rozental, 2016).

Aspergillus species can form biofilms, which are defined with the presence of a mycelium in the extracellular matrix, during different types of chronic *Aspergillosis* infections. The pathogenicity of *Aspergillus* is significantly influenced by its biofilm (Baron *et al.*, 1992). The development of biofilms, which promotes hyphae adherence to host cells and increases resistance to antifungal treatment, is primarily responsible for fungal virulence (D'Eça *et al.*, 2011). The aim of this study to isolate, identification and detection of virulence factors of *A. flavus* that increasing the degree of damage of the host as hydrolysis of albumin, hemolytic activity on blood agar and formation of biofilm in dogs.

MATERIALS AND METHODS

ISOLATION OF FUNGI

In Baghdad province (Al-Rusafa and Al-Karkh) Al-Rusafa district (Zayouna, Al-Mashtel Street, Adamiyah and Al-Karkh district (Baghdad Veterinary Hospital) during November 2023 to January 2024, fifty samples of nasal swab from infected dogs were taken from veterinary clinic and stray dogs from different ages between (2-5years), sexes (male and female) and breeds like (terriers, German shepherds and police dog) by using a sterilizing swab. All of the collected samples were stored in a sterilizer tube containing transport media at 4 °C in an ice container until they cultivated.

IDENTIFICATION OF FUNGI

The samples were cultured on Sabouraud Dextrose Agar (SDA) from (Himedia-India) containing 500 mg/L of Cefalexin, then incubated at 25 °C for 4–7 days for diagnosing macroscopically and microscopically. Furthermore, *Aspergillus* spp. were cultured on special medium Czapek Dox Agar (Oxoid-England) for appearance of pigmentation on reverse side of plates. The microscopic examination of *Aspergillus* spp. and other fungi were performed according to Baron and Finegold by putting one drop of lactophenol cotton blue stain on a slide and mixing it with a small part of the fungus by loop, then covering it with a cover slip, and examined by a light microscope under a 40X lens to determine the fungal elements microscopically.

DETECTION OF VIRULENCE FACTORS OF *ASPERGILLUS FLAVUS*

PROTEINASE PRODUCTION MEDIUM

This medium was prepared according to (Mahmoud *et al.*,

1989). Take 68 mL of a solution containing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.04 g, K, HPO 0.5 g, NaCl 1 g, yeast extract 0.2 g, glucose 4 g, and bovine serum albumin medium. The solution was sterilized using a Millipore filter (0.22 μm). After that, it was mixed with 140 ml of agar agar, sterilized in an autoclave at 121 °C under 15 pound/inch for 15 minutes, and put into sterile petri dishes. This medium was used to detect the ability of *A. flavus* to hydrolyze albumin, which was detected by the well diffusion method carried out one well was made in a proteinase production medium that contained bovine serum albumin in this well culture of *A. flavus* and incubated at 25 °C for 4–7 days.

MEDIUM FOR HEMOLYTIC ACTIVITY

Sabouraud Dextrose Agar medium used with 5% glucose and 5% sheep blood added as supplements as mentioned in (Bonassoli *et al.*, 2005). Hemolysis in the petri dishes was classified into low, moderate, and high during a maximum of seven days of incubation at 25 °C.

DETERMINATION OF BIOFILM FORMATION

Biofilm formation was assessed in sterile 96-well microplates. In a tube containing 2 ml of brain heart infusion broth (BHIB) medium with 0.25% glucose, the culture was placed using a loop, and the tube was then incubated at 37 °C for 24 hours. A final solution was obtained, and 200 μl was added to 96-well polystyrene microliter plates that were sterile. After placing covers on the microplates, they were incubated for 24 hours at 37 °C. Following incubation, the microplate was flipped to a blot and washed three times with PBS. Each well was then filled with 1% crystal violet, and it was incubate for 15 minutes. The microplate was once again washed three times with PBS following incubation. After adding ethanol to each well (Ogundijo, 2017), (ELISA) reader, the wells' 450 nm readings were obtained, and the OD was noted for each well.

STATISTICAL ANALYSIS

The SAS application, which stands for statistical analysis system, utilized to determine the effect of various variables on the research parameters. To compare means significantly and the least significant difference (LSD). In this study, the chi-square test was utilized to evaluate percentages 0.05 and 0.01 probability (SAS, 2018).

RESULTS AND DISCUSSION

ISOLATION OF FUNGI FROM RESPIRATORY INFECTION SAMPLES OF DOGS

This study found a high percentage of fungal isolates which reached (100%) were isolated from dogs collected from different locations within the Baghdad Governorate. Part of *Aspergillus* spp., which included *A. flavus*, *A. niger*, and *A. terreus* from these 50 isolates, were identified as significant

differences were found between these species at $P \leq 0.05$, as shown in (Table 1), and there were also significant differences between about 13 other types of important fungi (2 yeast, 11 mold) as shown in Table 2.

Table 1: The percentage of *Aspergillus* spp. isolated from dogs respiratory infection.

Aspergillus spp.	Isolation No.	Percentage %
<i>Aspergillus flavus</i>	9	42.8
<i>Aspergillus niger</i>	9	42.8
<i>Aspergillus terreus</i>	3	14.2
Total	21	100%
P-value	--	0.0476 *

* ($P \leq 0.05$).

Table 2: Distribution of sample study according to fungal isolation from dog's respiratory infection.

Fungal spp.	Isolation No.	Percentage %
<i>Absidia</i>	1	2
<i>Aspergillus flavus</i>	9	18
<i>Aspergillus niger</i>	9	18
<i>Aspergillus terreus</i>	3	6
<i>Candida</i> spp.	4	8
<i>Chrysosporium sitophilum</i>	2	4
<i>Cladosporium</i> spp.	1	2
<i>Curvularia</i> spp.	1	2
<i>Geomyces</i> spp.	2	4
<i>Geotrichum</i>	1	2
<i>Penicillium</i> spp.	12	24
<i>Phialophora</i> spp.	1	2
<i>Rhizopus</i>	2	4
<i>Rodetilla</i> spp.	1	2
<i>Trichoderma</i> spp.	1	2
Total	50	100%
P-value	--	0.0028 **

Note: There are significant differences at level ($P \leq 0.01$). ** ($P \leq 0.01$).

Aspergillus flavus causes a variety of superficial and invasive infections in both humans and other animal species and it is a soil borne fungus, which is ubiquitous in distribution. In the present investigation, the clinical and mycological observations conclusively established that dogs were suffering with fungal rhinitis due to *A. flavus* infection. (Steinbach *et al.*, 2012; Khodavaisy *et al.*, 2016; Susan and Aiello, 1998; Pal, 2007, 2017). The majority of molds are harmless but many can cause significant infections in humans and dogs. The main *Aspergillus* species that cause complications for dogs are *Aspergillus flavus*, *Aspergillus niger*, and *Aspergillus terreus*, yet several other species can also cause disease in dogs.

IDENTIFICATION OF *ASPERGILLUS FLAVUS* ISOLATES

Aspergillus flavus was isolated and identified from dogs by sub culturing on SDA at 25°C for 5-7 days. The colonies appeared as yellow green in color with fluffy in texture (Figure 1). A white mycelium that spread outward to cover the whole surface of the medium was the beginning of the colony expansion. As sporulation started, the white colony color gradually convert to yellowish green or dark green color of the conidia, which eventually covered the whole surface. This matched the findings of (Thathana *et al.*, 2017), who observed that the mycelia were white in color and that the reverse of the plates had cream and olive or dark green conidia bordered by a white ring.



Figure 1: Macroscopic appearance of *A. flavus* on SDA at 25°C for 7 days.

Furthermore, Czapek dox agar colonies initially had a yellowish green appearance before changing to an olive green over time, whereas the reverse side's color changed from creamy to yellow. The average colony diameter was 4-5 cm (Figure 2). These outcomes were consistent with (Aşkun, 2006; Gao *et al.*, 2007; Rodrigues, 2007; Saleemi *et al.*, 2017).

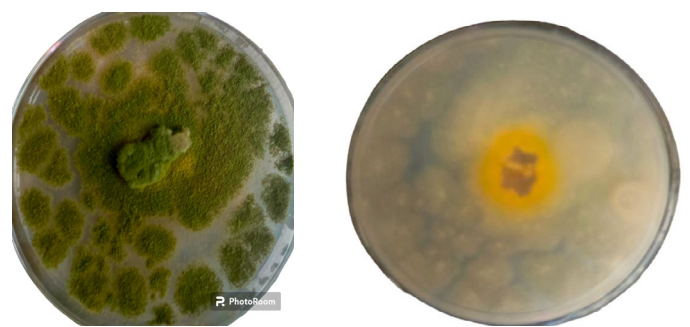


Figure 2: *Aspergillus flavus* on Czapek dox agar at 25 °C for 4-5 days.

The conidial head appeared complete flower (complete circle phialides) which were arranged as long conidiophores (Figure 3). The microscopic characteristics were in harmony with the *A. flavus* characteristics previously described (Diba *et al.*, 2007; Okayo *et al.*, 2020).

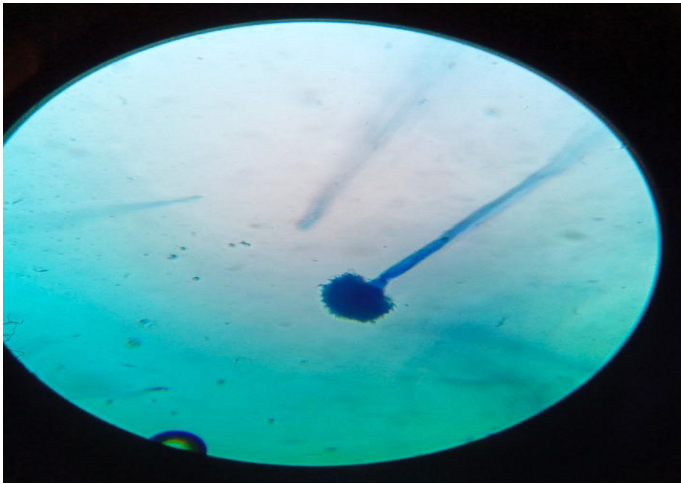


Figure 3: Microscopic appearance of *A. flavu* using stain lactophenol cotton blue (40x).

VIRULENCE FACTORS OF *A. FLAVUS*

Characteristics of virulence factors that facilitate fungal growth, survival, and spread in tissues of mammals and cause illness (Hoog *et al.*, 2000). This study measured the diameter of the albumin inhibition zone as shown in (Table 3) and (Figures 4, 5), to determine the degree of virulence (higher, moderate, weak, and very weak) of nine isolates of *A. flavus* from fifty samples of nasal swab. The findings demonstrated that each isolate of *A. flavus* was able to degrade albumin protein, with highly significant differences in the degrees of virulence. Similar results were obtained from (Rodrigues *et al.*, 2005). The explanation for these observations was the ability of albumin to influence *Aspergillus* conidial germination and hyphae formation, particularly in *A. flavus* (Mezher and Bandar, 2016).

Table 3: The results of proteinase action by *A. flavus*.

Degree of virulence	Isolated <i>A.flavus</i>	Percentage (%)	Diameter of inhibition zone(mm)
Higher	A and B	11.11	29
Moderate	C, D, E, G and I	27.7	20
Weak	H	0.55	15
Very weak	F	0.55	10
P_value	---	0.0387 ($P \leq 0.0001$ ($P \leq 0.01$), 0.05),	

Note: There are high significant differences at level ($P \leq 0.05$) and ability to albumin hydrolyze by *A. flavus* at level ($P \leq 0.01$).

Likewise *A. flavus* isolates showed positive action of hemolysis on blood agar. The research showed that all isolates of *A. flavus* had hemolytic action on Blood Agar

with significant differences between level of hemolytic ($P \leq 0.05$) classified into weak, moderate, and high as shown in (Table 4) and (Figures 6, 7). Hemolytic action is taking into account one of the most significant virulence factors, a transparent or semi-transparent zone surrounding the inoculation site was determined to be positive for hemolytic action. Hemolysin lyses red blood cells (RBCs) by creating pores or holes in their membranes, which releases iron and encourages the growth of microorganisms (Pasqualotto, 2009). The present results are consistent with previous studies by (Bavadharani *et al.*, 2022; Satish *et al.*, 2013; Savković *et al.*, 2022).

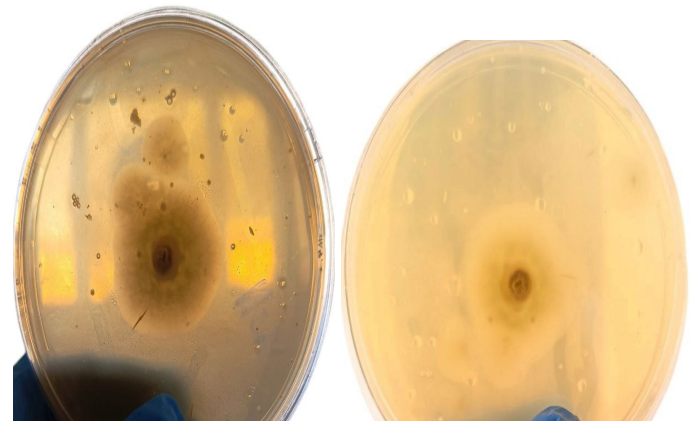


Figure 4: Hydrolysis of albumin by *A. flavus* in proteinase production medium.

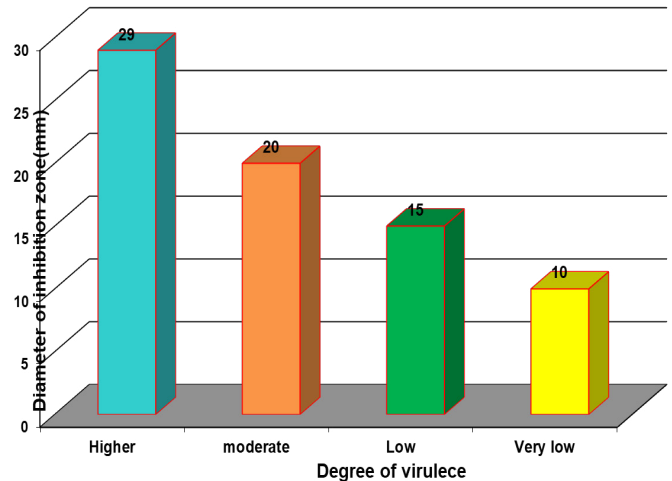


Figure 5: Measurement the diameter of inhibition zone in albumin medium.

Table 4: The results of Hemolytic activity by *A. flavus*.

Isolates of <i>A. flavus</i>	Degree of hemolysis	Percentage (%)
Two isolated	High	11.11
Five isolated	Moderate	27.77
Two isolated	Weak	11.11
P value	--* ($P \leq 0.05$)	0.0316

Note: the significant differences between level of hemolytic ($P \leq 0.05$).

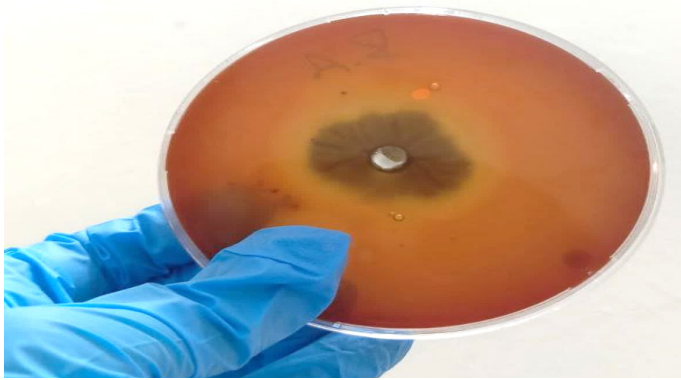


Figure 6: Hemolytic action by *A. flavus* on blood agar.

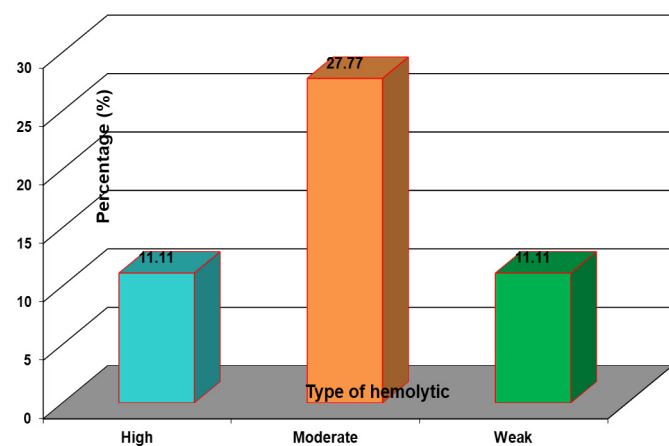


Figure 7: Results of hemolytic activity of *A. flavus*.

Another virulence factor of *A. flavus* was biofilm formation. All isolates of *A. flavus* biofilm formation were classified as weak, moderate, and high. The biofilm mass was higher in (A, B, and C) isolates of this mold with high significant differences ($P \leq 0.01$), as shown in (Table 5 and Figure 8), respectively. This result was in agreement with other studies (Ogundijo, 2017). The reason behind these results may be attributable to the ability of *A. flavus* to utilize more protein. This result is similar to the biofilm formation by *A. flavus* reported in a previous study by (Ghorbel *et al.*, 2019). The study reported that *A. flavus* utilized more serum proteins in the production of biofilms. This could suggest that a riskier factor in *A. flavus* is biofilm, which has been associated with infections of the upper respiratory tract (Ramage *et al.*, 2012; Wuren *et al.*, 2014).

Table 5: Biofilm formation by *A. flavus*.

Isolates of <i>A. flavus</i>	Degree of biofilm formation	Percentage (%)
Three isolates	High	33.3
Four isolates	Moderate	44.4
Two isolates	Weak	22.2
P value	--($P \leq 0.01$)	0.00839

Note: high significant differences between isolation at level ($P \leq 0.01$).

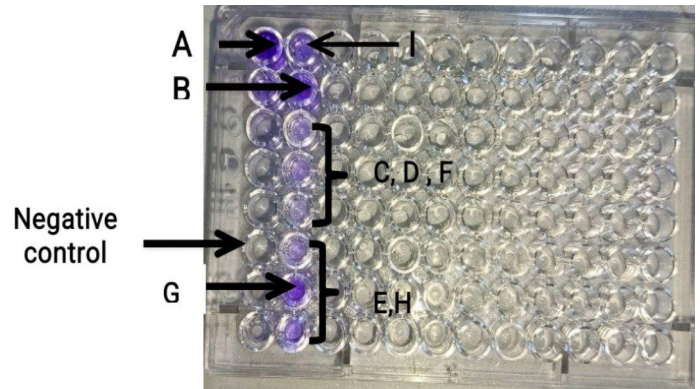


Figure 8: Microplate inoculated with nine *A. flavus* isolates with negative control for detection of biofilm formation result show high Biofilm formation in sample A, B and G, moderate in sample C, D, F and I and weak biofilm in sample E and H.

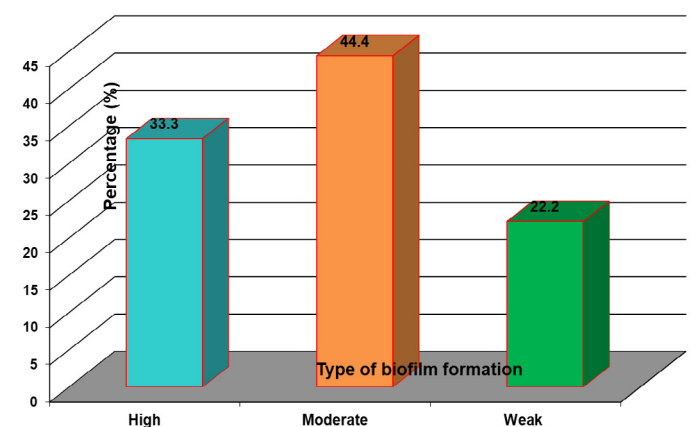


Figure 9: Biofilm formation by *A. flavus*.

CONCLUSIONS AND RECOMMENDATIONS

Any microorganism's virulence factors are crucial in distinguishing harmful from non-pathogenic species. The invasiveness of the *Aspergillus* species may be indicated by the virulence factors found in isolates of the fungus. The study determined that the three most significant virulence factors of *Aspergillus flavus* isolated from dogs are proteinase, hemolysin, and biofilm, with respect to the variations in virulence factors generated by *Aspergillus flavus*.

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The novelty of the study is focus on isolation and determination the virulence factor of *A. flavus* (specially biofilm formation) that has capacity to cause disease even in healthy animals without immune suppression, and these are life threatening.

AUTHOR'S CONTRIBUTION

Each of these writers made an equal contribution.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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