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Infection of *Aspergillus flavus* in Brain Tissues of Albino Male Rats

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Abstract

Aspergillus flavus is considered a one of the fungi which can cause dangerous fungal infections for humans and animals. In this study, an isolate of this fungus was identified according to the morphological and molecular methods. The polymerase chain reaction (PCR) was used to confirm the identification of the isolate appeared genes of the *A. flavus* (*FLA*) and a putative RNA-Pol II transcription elongation factor (*RtfA*) which located in 497 and 263 base pairs (bp), respectively**.** Experimentally, two groups of the albino male rats were used in this study by which one group was immunosuppressed and infected with 0.5 ml containing 10^6 spores of *A. flavus* intra-peritoneally while other group was inoculated with 0.5 ml of the sterile normal saline. Brains of the infected rats revealed lesions that the brain appeared swelling and congestion of the cerebrum pushing the cerebellum down. Cerebral cortex with prominent haemorrhage was illustrated with lymphocytic infiltration. Also, demonstrating pyogenic granuloma in the cerebral cortex composed of centre of caseous necrosis surrounded by massive neutrophils and other chronic inflammatory cells. In addition, the brain sections possessed multiple multinucleated giant cells and dissemination of *A. flavus* into ventricular space and choroid plexus.

Keywords: *Aspergillus flavus*, Albino male rats, Brain tissue.

Introduction

Aspergillus flavus are considered pathogen can cause diseases for humans, animals, and plants [1-3]. These fungal diseases are opportunistic mycoses which emerged if a host suffered from weak [4-5]. The impact of *Aspergillus* species on immune-compromised individuals cannot be overstated immunity, as it continues to be a significant cause of life-threatening infections. This group of vulnerable patients includes those who have prolonged neutropenia, solid organ transplant, undergone allogeneic hematopoietic stem cell transplant, and those with inherited or acquired immune-deficiencies, as well as those who have been prescribed corticosteroids, among others [6].

Aspergillus flavus is a one of the causative agents that cause the mycoses [7]. This fungus has most importance in the public health including veterinary aspect because *A. falvus* possesses ability to cause diseases especially its aflatoxins which lead to occur food poisoning in the farms [8-9]. *A. flavus* is a prevalent organism that leads to a wide range of clinical conditions, which can be broadly classified into four categories: first: allergic infections, second: invasive aspergillosis, third: posttraumatic infections, and fourth: sub-acute/chronic infections. With the exception of the fourth group, the lungs are the most frequently affected organs in all other categories, followed by the peripheral and central nervous system, CNS [10]. Therefore, this study aimed to demonstrate effects of *A. flavus* on the brain tissue of albino male rats.

Material and Methods

Identification of *Asperigillus flavus*

Asperigillus flavus grew on the Sabouraud dextrose agar (SDA) at 37 ° C for 5 days. This fungus was isolated from respiratory sources (septum) and identified morphologically depending on the macro and microscopic characteristics. e.g., color, shape, and size of the colonies, exudates, appearances of a microscope. The appearances were performed using lactophenol cotton blue (LCB) stain and examined under microscope (10 and 40 X) to reveal conidia, conidiophore, vesicles, and hypha. In addition, PCR technique for confirming the morphological identification. This technique was used to detect *FLA* genes in the DNA of *A. flavus* which was extracted according to instruction of the Genoid kit. These genes were identified using primers of *FLA* were forward primer: 5'-

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GTAGGGTTCCTAGCGAGCC-3' and reverse one: 5'- GGAAAAAGATTGATTTGCGTTC-3' [11]. The PCR process was obtained using conditions were initial denaturation at 95°C for 5 min. 1 cycle, denaturation at 95°C for 45 sec., annealing at 62°C for 45 sec. 27 cycle, and elongation 72°C for 45 sec. and final extension at 72°C for 10 min. 1 cycle. Also, *RtfA* genes were detected using forward primer: 5′-GTTCCCTTTCGTTGCTTGTTCAGACTC- 3' and reverse primer: 5′-CAGTCGACTTGGTGTCCAGTGATCC-3'. These genes have length is 263 bp [12]. The genes were to detect a virulence of this fungal isolate selected isolate which was used for experimental infection. The PCR reactions occurred at same conditions of *FLA* primers, except the annealing temperature was at 64 ºC for 1 minute and the quantities PCR mixture was shown in (Table 1).

Experimental infection

Spore solution was prepared using adding amount of the sterile normal saline on a plate containing 5 days old SDA *A. flavus*. Then, spores were gently harvested and placed in a sterile tube containing 9 ml of that saline with 1 ml of the Tween-20 solution [13]. The spore yield was washed twice and re-suspended by the same solution. The percentage of the spore viability were enumerated directly through 40 X magnification by Neubauer hemocytometer [13] and using Trypan blue stain (TBS). The final percentage was 10^6 viable conidia/0.5ml [14]. Albino male rats which were well managed [15] and used in this process of the infection. Animals were divided into two groups in which each group contained 10 members which were kept in cages separately. One group was considered negative (without infection) categories while others were positive (infection) group. Ages and weights of these animals ranged 8- 10 weeks and 200-250 grams, respectively. Prior infection test, animals of the positive group were subcutaneously and daily injected with 0.5 ml of the cortisone (2.5 mg / ml) for 6 days to obtain immunosuppressed animals. After completing six days, animals of the positive group were intra-peritoneally inoculated with 0.5 mL contained 10^6 spores of *A. falvus* while negative group was inoculated with 0.5 ml of the normal saline only at the same rout. The infected animals were injected with 0.5 ml of the levofloxacin (200µg / ml) subcutaneously and daily during all days of the fungal infection to prevent the bacterial infection. This infection process was performed according to [14] and all animals including negative group were left for 20 days. Then, rats were sacrificed and their brains were selected and prepared to the pathological sections. The periodic acid Schiff (PAS) stain was used to reveal fungal elements in the brain tissue while hematoxylin and eosin (H&E) stain used for appearing pathological changes in that tissue.

Results

Identification of Aspergillus flavus

Aspergillus flavus produced flat, wrinkled, and green colony having white edges on SDA and reverse was brownish yellow. Under microscope, a slide stained with LCP stain revealed oval conidia, conidiophores possessing vesicles on their tops (Fig. 1). In addition, PCR detected genes of the *FLA* genes and RtfA which located in 497 and 263 base pairs (bp), respectively (Fig. 2).

Pathological changes

Gross examination of the brain revealed severe cerebral swelling and congestion (Fig.3) in the infected rats compared with animals of the negative group has normal brain size or with mild changes. The microscopic examination illustrated cerebral cortex lesions which possessed prominent hemorrhage with lymphocytic infiltration besides hyphae of *A. flavus* were noticed in the brain (Fig. 4). In addition, different types of inflammatory cells such as macrophages and neutrophils were also demonstrated (Fig. 5). The cerebrum of the positive group illustrates typical pyogenic granuloma in cerebral cortex composed of center of caseous necrosis surrounded by massive neutrophils and other chronic inflammatory cells and also present multiple multinucleated giant cells (Fig. 6).

Discussion

Aspergillus flavus has been considered a one of the most important fungi which can cause mycoses. Although the fungus infects lungs commonly but it can reach brain tissues [4]. The present study showed invasiveness of *A. flavus* in the brain tissues (Fig.5) of the albino male rats which were tested in the current study. This fungus is able to attack different tissues including brains that the severity of the infection appeared in the animals which are immunocompromised [1]. This evidence is agreed with results of the present study that appeared lesions in the cerebral cortex, cerebral lateral ventricle, and other parts of the rat brains (Fig.6). Interestingly, the infected animals were injected with cortisone before days of the infection process to get these animals have weak immunity. Also, *Aspergillus* species can produce metabolites are immune-suppressed lead to a host is adapted for the infection as well as the infection of the central nervous system can be obtained through crossing the fungus to the blood – brain barrier (BBB). Decreasing oxygen, edema, and alveolar flooding can be formed and produce the obstructions which make the *Aspergillus* species to promote an environment is hypoxic. This environment helps these fungi to be survivals in the tissues that have inflammation [16]. Depending of these obstructions, it may be the experimental albino

rats of the current study suffered from hypoxic condition which predispose *A. flavus* to invade the brain tissues. In addition, aflatoxin B1 (AFB1) of *A. flavus* has important role in the invasion of this fungus such as brain infection by which neuro-inflammation and neurotoxicity occur. These brain and nervous lesions are obtained due to active soluble epoxide hydrolase which increases in the brain [17,18]. As well as the mycotoxin can cross the BBB to cause pathological changes. In other words, brain cells suffer from degeneration during few minutes if oxygen supply does not provide the brain sufficiently and the AFB1 has effects on concentrations of the rat brain neurotransmitter [19]. This may explain why infected rats of the present study suffered from infection of *A. flavus* in their brains.

Previous study supported the effects of AFB1 on the brain by which a suitable dose of this mycotoxin revealed disturbances of the immune regulation were noticed in the neurodegenerative disorders. In addition, the AFB1 induced production cytokines such as IL-6 [20]. Among innate immunity, microglia have remarkable role for protection the CNS from infections including mycoses. This immune defense appears in a process of the neural parenchyma inflammation that a toxicity of neurons occurs. The toxicity is noticed when microglia have uncontrolled activity leading to produce immune substances example, IL-6 [21-23]. The brain tissues of a rat fetus had abnormal lesions according to the study of [24] that resulted from AFB1. In humans, *A. flavus* infection occurs in patients either immunocompetent or immunosuppressed ones. This infection spreads brain and cause lesions in a parenchyma of the brain due to defective cellular immunity. The brain lesions were secondarily resulted in fungal blood spread that reaches the brain [25]. Contextually, study demonstrated the brain of a patient infected with *A. flavus*. and the nasal sinuses were evolved. Treating this case was performed by isavuconazole during long course [6, 26].

Cerebral aspergillosis was noticed and this infection resulted in abscesses and necrosis in the brain through disseminating blood stream. Also, hemorrhages and congestions were observed in the brain due to aspergillus infection [14, 27]. Other researchers showed the brains are infected with *Aspergillus* species leading to reveal multifocal suppuration and congestion appearance as well as necrotic and hemorrhagic foci observed on the cortex of the cerebrum and cerebrum[28]. Another study used experimental model animals to infection of *Aspergiillus* species which appeared brain lesions such as hemorrhage and abscess [29]. Also, researchers showed that the CNS mycoses increased and led to high ratios of the morbidity and mortality. In addition, the immune responses were stimulated by these mycoses [30- 31]. Neurotransmitter is decreased due to AFB1 leading to affect final protein products besides process of metabolizing amino acids. This mechanistic step makes hyper- ammonia which pass BBB easily for getting glutamate neurotransmitters which are considered cytotoxic for brain tissues and finally encephalopathy occurs. Precisely, AFB1 of *A. flavus* is related to the nervous symptoms and brain lesions especially in the rats [8, 24, 32].

Furthermore, it may be peroxidation of the lipid increased due to the AFB1 which participates in the cytotoxic damage of the male rat brain and then neurodegenerative diseases occur [23]. According to the mentioned words, results of the current study were agreed with these previous studies that the lesions in the brain tissues occur due to *A. flavus*. It may be these infected rats stimulated this fungus to make the animals possessed uncontrolled active microglia which represented another predisposing factor to infect the brain tissues of these animals. Additionally, *A. flavus* invaded brain (Fig.5) which may be attributed to growth of this fungus in the animal host that leading to production of AFB1 causing abnormal changes in the brain. In this context, the invading brain indicated the fungus cross the BBB. This result indicated the fungal isolate of this study is pathogen and virulent.

Conclusions and Recommendations

Aspergillus flavus has an ability to attack brains of the immunosuppressed albino male rats and the fungus is considered most dangerous pathogen due to its aflatoxins and contamination of the food and crops. The present study recommended further more studies to detect *A. flavus* in the CNS of human and animal patients including those have brain stroke, meningitis, cancer, and renal failure.

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Conflict of interest

There is no conflict of the interest in this scientific article.

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TABLE 1. Amounts of the molecular mixtures used in the PCR process.

Substances	*FLA* gene primers (µL)	*RTFA* gene primers (µL)
Premix	5	5
DNA template	1	2
Forward primer	1	1
Reverse primer	1	1
Distilled water	12	11

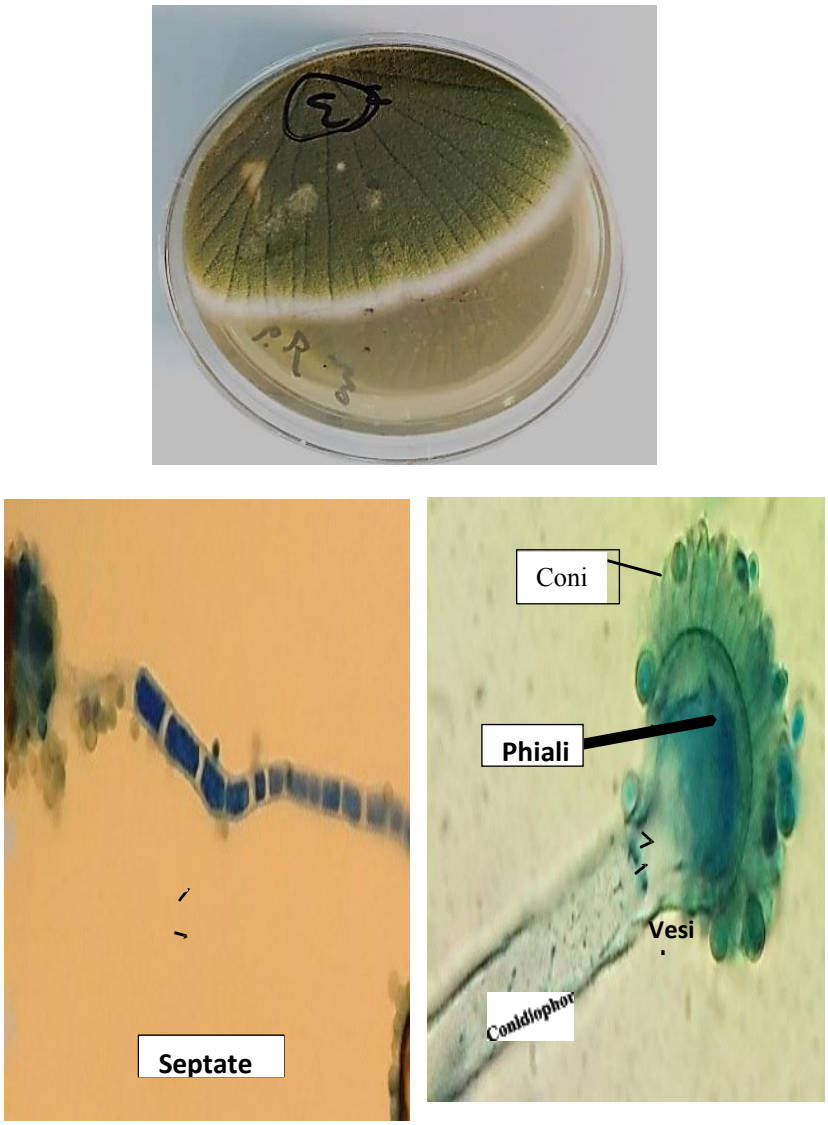

Fig. 1. Macro and microscopic morphology of 5 old days *A. flavus* growing on SDA at 37 C°.

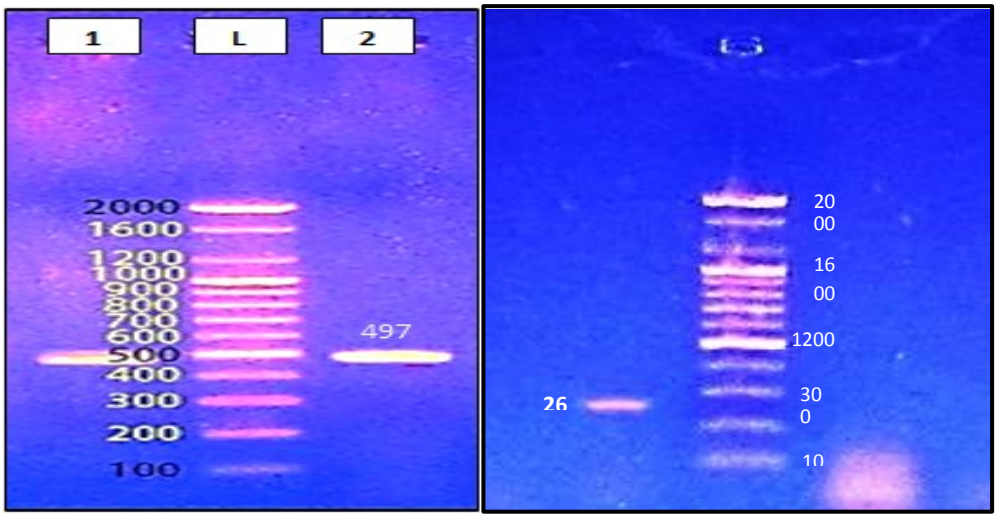

Fig. 2. Ethydium stained agarose appears: Right, *FLA* genes located at 497 bp: 1 and 2 lanes were positive isolates of *A. flavus* while L represented DNA marker (2000-100bp). Left, RtfA gene of *A, flavus* located at 263 bp.

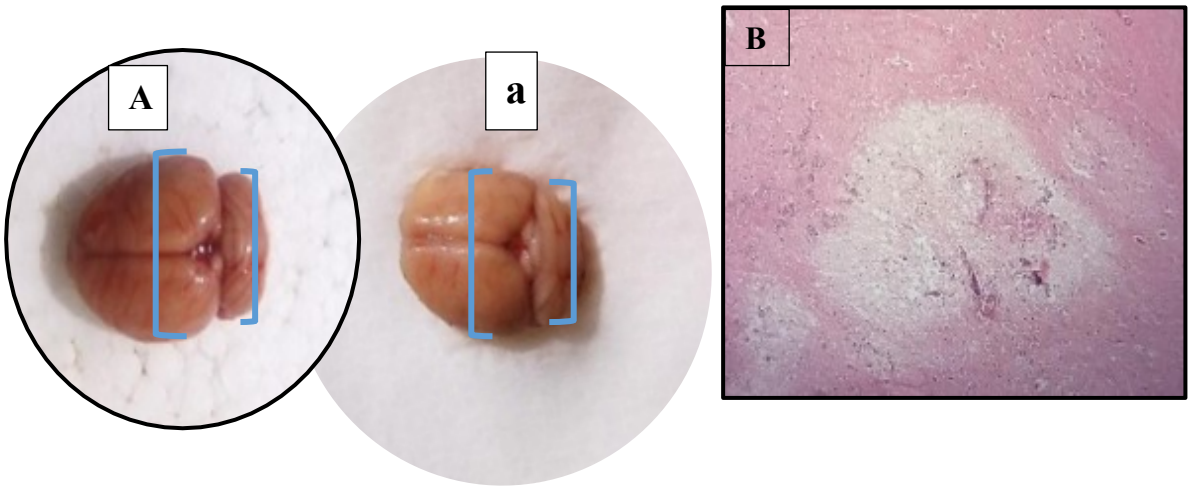

Fig. 3. A: Gross image of brain of positive group shows prominent swelling and congestion of cerebrum pushing the cerebellum down compared with the normal uniformed one (a) of control group. There is clear variation in ratio of cerebellum size to cerebrum size . B: Histopathological section of cerebral cortex of the infected rat. In the center: Focus of liquefactive necrosis due to *A. flavus* dissemination and invasion.

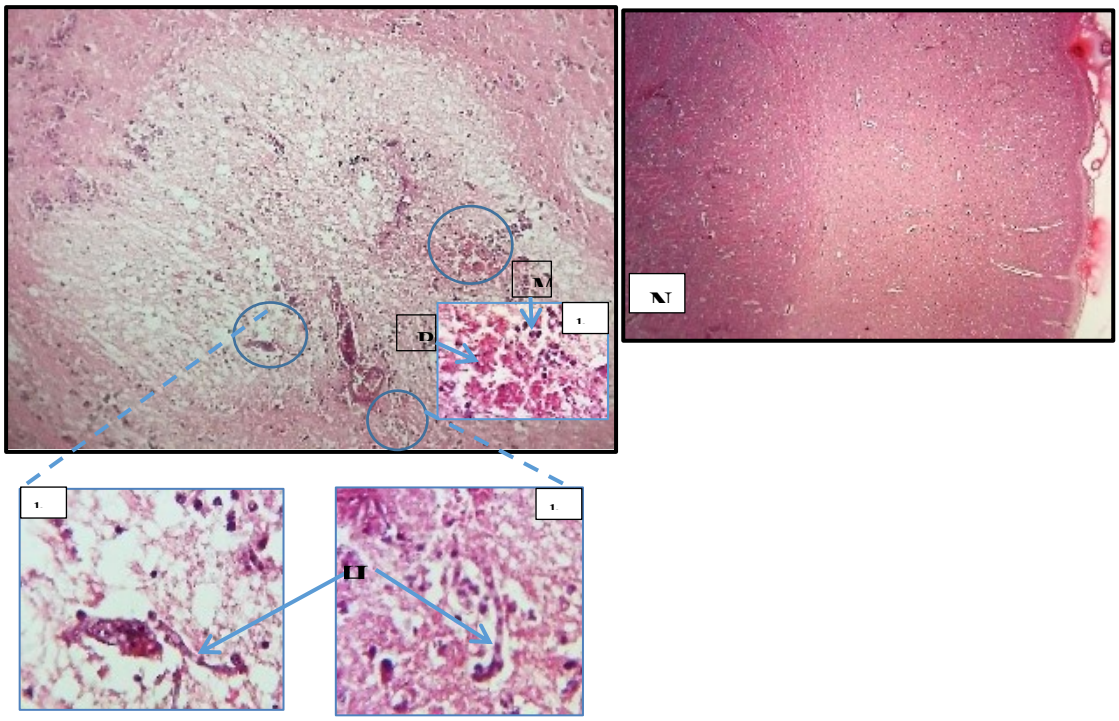

Fig.4. B2: Histopathological section of cerebral cortex 100X. There is prominent hemorrhage (R) with lymphocytic (M) infiltration (b1). In the lower (b2& b3), high magnification (40X) illustrates branched and septate hyphae of *A. flavus* . N. represents the normal histological section of cerebral tissue.

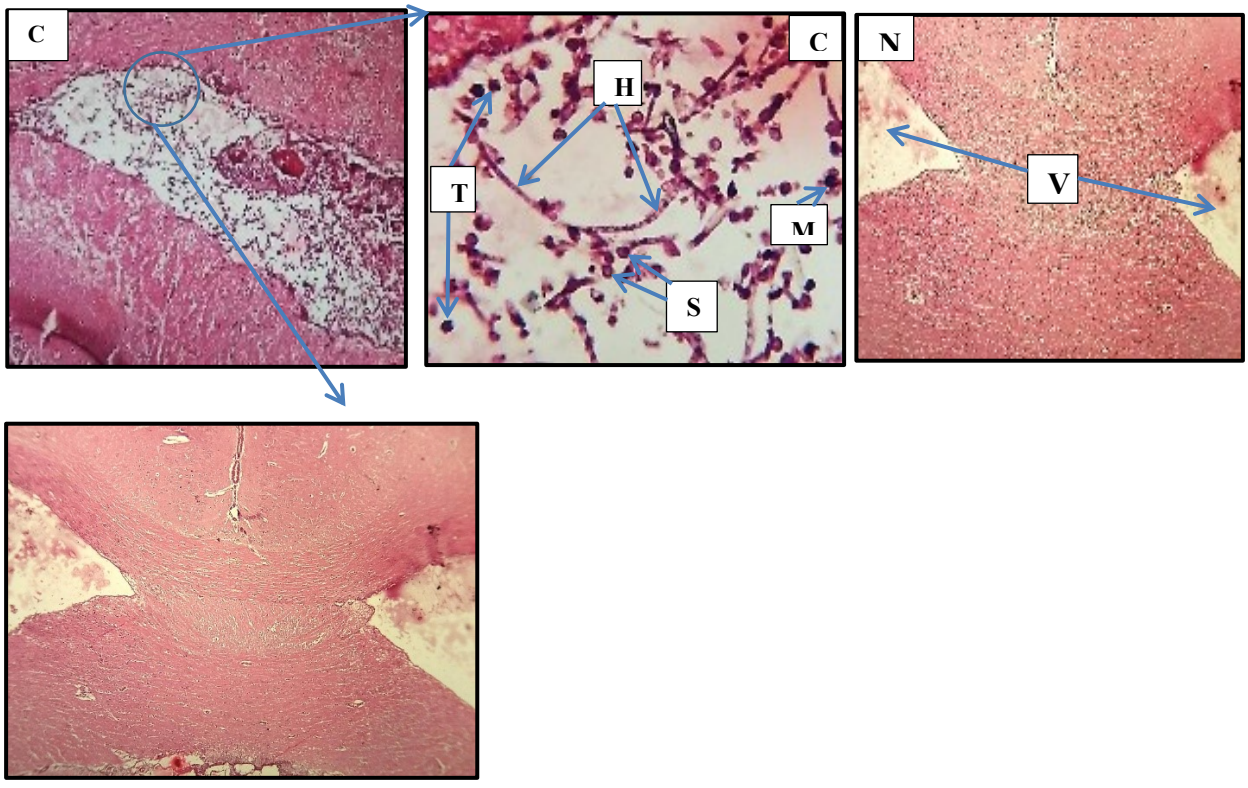

Fig.5. C1: Histopathological section of cerebral lateral ventricle for positive group shows dissemination of *A. flavus* into ventricular space and choroid plexus. C2: Histopathological section of the same field illustrates the branched and septated fungal hyphae (H) and spores (S) with different types of inflammatory cells such as macrophages (M) and neutrophils (T). N. Normal cerebral tissue and ventricles (V), 40X.

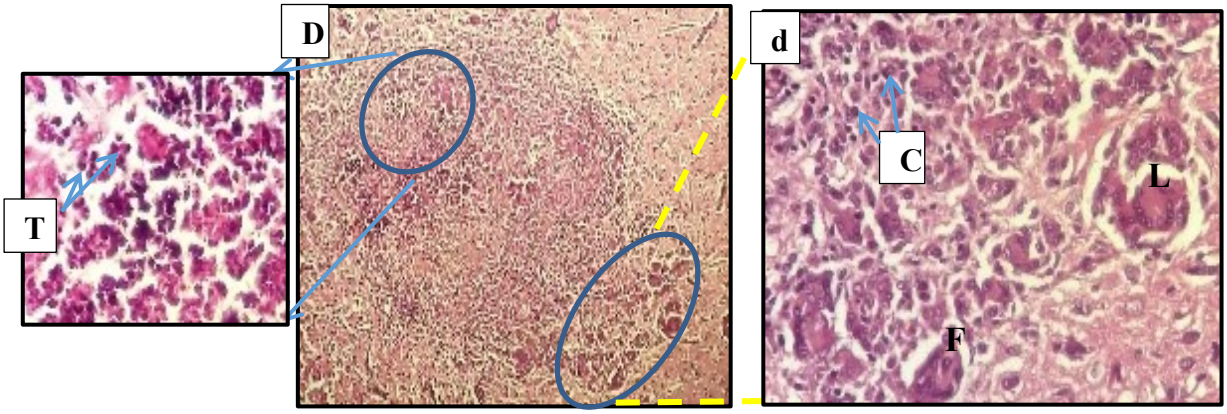

Fig.6. D. Histopathological section of cerebrum of the positive group illustrates typical pyogenic granuloma in cerebral cortex composed of center of caseous necrosis (N) surrounded by massive neutrophils (T) (100X). d. High magnification of the same field focusing on multinucleated giant cells Langhans giant cells (L), foreign body giant cells (F), and chronic inflammatory cells (C). 40X.

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اصابة فطر الرشاشيات *Aspergillus flavus* لنسيج ادمغة الفئران الذكور البيضاء

ضرغام على الحسن

قسم الأحياء الدقيقة - كلية الطب البيطري - جامعة الشطرة – العراق.

المستخلص

يعتبر فطر الرشاشيات *Aspergillus flavus* واحد من الفطريات التي تسبب امراضا خطيرة للإنسان والحيوان. في هذه الدراسة، عزلة من هذا الفطر كانت قد شخصت مظهريا وجزيئيا حيث استخدم نوعان من البادئات تم من خلالهما تشخيص المورثين وهما : *FLA* و RtfA ضمن الحمض النووي منقوص الاوكسجين في الفطر المشار اليه. ايضا، في هذه الدراسة كانت قد استخدمت مجموعتان من الفئران الذكور البيضاء وقد احتوت كل مجموعة على عشرة فئران. هيئت احدى المجموعتين التي اعتبرت مكانا لتجربة الاصابة وضعفت مناعتها قبل عملية الاصابة بينما اعتبرت الاخرى مجموعة سيطرة. حقن كل حيوان من مجموعة التجربة بنصف مل احتوى على 10^6 بوغ من فطر الرشاشيات في حين حقنت حيوانات مجموعة السيطرة بنفس الكمية لكن بمحلول معقم من الملح الطبيعي غير المحتوي على اي بوغ. تركت الحيوانات جميعها لمدة 20 يوما وبعد انتهاء المدة الزمنية لعملية الاصابة، قتلت تلك الحيوانات ثم انتقيت ادمغتها لمعرفة التغيرات النسيجية التي تسبب بها الفطر اذ خضعت لعملية التقطيع النسيجي واتضح ان حيوانات تجربة الاصابة اظهرت تغيرات نسيجية مرضية بالمقارنة مع حيوانات السيطرة. لوحظ هناك نزف واحتقان في ادمغة الحيوانات وتغيرات ذات مظهر شبيه بالأجبان وتأثر قشرة الدماغ. كذلك تم تشخيص الورم الحبيبي فضلا عن وجود تنخر محاط بخلايا التهابية وخلايا عدلة. بالإضافة الى ذلك، لوحظت الخلايا العملاقة في انسجة ادمغة الحيوانات المصابة وان الفطر تموضع في تلك الانسجة.

الكلمات الدالة: فطر الرشاشيات، الفئران الذكور البيضاء، نسيج الدماغ.