10.21608/avmj.2025.324195.1412 Assiut University web-site: www.aun.edu.eg

# MOLECULAR DETECTION OF *ASPERGILLUS NIGER* GENES NIG1, CMDA AND MYCOTOXIN GENES FUM1, PKS FROM OTOMYCOSIS AMONG BREEDERS OF DOMESTIC CATS WITH SPECIAL EMPHASIS TO RISK FACTORS IN DIYALA GOVERNORATE, IRAQ

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**Received:** 4 December 2024; **Accepted:** 12 February 2025

## ABSTRACT

*Aspergillus* spp. is saprophytic opportunistic fungi that are a leading cause of otomycosis in human. Isolation of *Aspergillus niger* (*A. niger*) from external ear of domestic cats breeders (DCB), molecular characterization of *A. niger* by conventional polymerase chain reaction (PCR), detection of putative risk factors for infection with *Aspergillus*, detection of fumonisin and ochratoxin by PCR. One hundred and six external ear swabs were taken from DCB attended to private veterinary clinics and cultured on Sabouraud dextrose agar. Fungal growth was identified as *A. niger* according to cultural characteristics. All *A. niger* positive samples were subjected to confirmatory step by conventional PCR using species-specific PCR primer pairs for *A. niger* (NIG1) and calmodulin (Cmd A). Production of fumonisin and ochratoxin by *A. niger* confirmed by PCR using specific primers. *A. niger* was isolated from 10.38% ear swabs of DCB. Significant correlation was reported between age group, season, absence of earwax, using of antibiotics eardrops and isolation of *A. niger*. No significant correlation was recorded between sex, education levels, economic status, swimming, exposure to trauma, diabetes miletus, occupation and isolation of *A. niger*. Fumonisin gene (FUM1) was detected in 63.64% (7/11) of *A. niger* isolated from ear of DCB. Ochratoxin gene (PKS) was not produced by any isolates of *A.niger* , 0% (0/11). Using of NIG1 and Cmd A has equal significance in molecular diagnosis of *A. niger*. Age group, season, absence of earwax, using of antibiotics eardrops have serious effect on infection of ear with *A. niger* among DCB. Fumonisin is the major mycotoxin produced from *A. niger in* otomycosis. *A. niger* ear infection represents significant problem among domestic cats breeders.

***Keywords:*** *Aspergillus niger*, Otomycosis, Domestic Cat Breeders, Fumonisin, Ochratoxin, Risk factors

## INTRODUCTION

More preventive techniques are needed since some fungal infections, whether they are caused by actual pathogens or by opportunists connected to the spread of zoonoses, are disregarded in public health initiatives (Mokhtar, 2022). Because the infected fungi did not adapt adequately to human host environment, immunocompromised patients may experience a severe immune response that is potentially lethal (Zhang, 2015).

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It is estimated that there are over 200 named species of *Aspergillus*. *A. niger* is part of a group of species that are collectively referred to as the *Aspergillus* section *Nigri*, which was previously designated the '*Aspergillus niger* group'(Riedling *et al.*, 2024). A distinguishing characteristic of all species within this filamentous fungus is the presence of black conidial heads. *Aspergillus* species is capable of causing infections in individuals with intact immune systems and those with compromised immune systems (Raj *et al.*, 2025).

A fungal infection of the ear called *Aspergillus* otomycosis is brought on by members of the *Aspergillus* genus. Although people and dogs are more commonly known to have this ailment, cats can also get it, but it happens infrequently. The external ear canal is the primary site of the infection, which can cause discomfort, inflammation, and additional consequences if treatment is not received (Medleau & Hnilica, 2006). Cats affected by *Aspergillus* otomycosis may present with a range of symptoms, including; head shaking or scratching at the affected ear, ear discharge, which may be waxy, purulent, or contain fungal debris, a foul odour emanating from the ear, erythema and swelling of the ear canal, pain or sensitivity when the ear is touched; and hearing loss in severe or chronic cases (Miller *et al.*, 2012).

It is an established fact that the ability of pathogenic *Aspergillus* strains to infect the host is contingent upon the presence of virulence factors (Bavadharani *et al.*, 2022). The production of virulence factors by *Aspergillus* species is a crucial aspect of the infection process, contributing to its establishment and, in subsequent stages, potentially leading to invasive or disseminated infections (Sales-Campos *et al.*, 2013). The virulence factors of the pathogen include biofilm production, as well as lipase, phospholipase, amylase, hemolysin, proteinase, esterase, and elastase (Bavadharani *et al.*, 2022). Biofilm formation is a pivotal element of fungal virulence, primarily by promoting the adherence of hyphae to host cells and enhancing resistance to antifungal agents. A variety of extracellular enzymes produced by *Aspergillus* species facilitate the breakdown of complex polysaccharides into simple sugars. These sugars are then assimilated by the host organism for use in growth, reproduction and survival (Bavadharani *et al.*, 2022).

Every year in the all the world, people who come into touch with pets like dogs and cats get a wide range of infections, from deadly systemic diseases to superficial cutaneous ailments including otitis (Al-Ezzy *et al.*, 2017; Jameel & Al-Ezzy, 2017). The most prevalent diseases linked to pets are most likely fungal skin infections (skin dermatophytosis or other skin disorders) in contact with cats and dogs. Numerous zoonotic illnesses can transfer from pets to people. Because many zoonotic infections are either underdiagnosed or not reported to health authorities, the breadth of the issue is not well established (Rabinowitz *et al.*, 2007).

Several risk factors can contribute to the development of otomycosis in cat owners. Below are the effects of different risk factors such as:

**A) Exposure to Fungal Pathogens from Cats:** Cats can carry fungal pathogens such as *Aspergillus* or *Candida* species, which are common causes of otomycosis. Close contact with cats, especially those with ear infections or skin conditions, increases the risk of transmission to humans (Gupta, 2015).

**B) Poor Ear Hygiene:** Cat owners who neglect ear hygiene or use improper cleaning methods (e.g., cotton swabs) may damage the ear canal's protective barrier, making it more susceptible to fungal infections (Bojanović *et al.*, 2023).

**C) Humid Environments:** Cat owners living in humid climates or those who frequently expose their ears to moisture (e.g., during bathing or swimming) are at higher

risk of otomycosis. Fungal pathogens thrive in warm, moist environments (Ho, Vrabec, *et al.*, 2006).

**D) Immunocompromised States:** Cat owners with weakened immune systems (e.g., due to diabetes, human immunodeficiency virus (HIV), or long-term corticosteroid use) are more prone to fungal infections, including otomycosis (Anwar & Gohar, 2014).

**E) Use of Antibiotics or Steroid Ear Drops:** Prolonged use of antibiotic or steroid ear drops can alter the ear canal's microbial balance, and promoting fungal overgrowth (Vennewald & Klemm, 2010).

**F) Allergic Reactions to Cat Dander:** Cat owners with allergies to cat dander may experience chronic inflammation or itching in the ear canal, which can lead to scratching and secondary fungal infections (Satish, 2011).

**G) Occupational or Behavioral Factors:** Cat owners who work in environments with high fungal exposure (e.g., veterinarians, and pet groomers) or those who frequently clean litter boxes may be at higher risk of otomycosis (Pontes *et al.*, 2009).

Benefits to a pet's emotional, physical, and social well-being; yet, domestic animals can spread zoonotic infections. According to a comprehensive survey, around 75% of families have intimate relationships with their pets, which includes face-licking and sleeping in the owners' beds (Stull *et al.*, 2012). According to more research, people who are at high risk of developing diseases linked to pets may not be aware of the hazards involved in owning pets or suggestions for lowering those risks. For instance, 77% of people who raise pets receive a cancer diagnosis after purchasing a high-risk animal (Stull *et al.*, 2014).

Current study aims to isolation of *A. niger* from external ear of cats' breeders, molecular characterization of *A. niger* by conventional PCR, detection of putative risk factors for infection with *Aspergillus*, and detection of fumonisin and ochratoxin by PCR.

## MATERIALS AND METHODS

### 1. Ethical Consideration

Current randomized experimental procedures were performed in accordance with the guides of Helsinki declaration and confirmed by the ethics committee at pathology department, college of veterinary medicine, University of Diyala Iraq. The approval No: PD, CVM-UOD-25 /202.

### 2- Area of the study

Current study was achieved in veterinary clinics at Diyala governorate and its capital city, Baqubah northeast Baghdad, Iraq

### 3- Collection of samples

Samples were collected from 10th September 2023 until 25th April 2024. The study included 106 DCB suffered from otomycosis. Swabs from external ear were taken and transfer to mycology laboratory at the college of veterinary medicine, University of Diyala.

### 4- Isolation of *A. niger*

Swabs were streaked into Sabouraud's dextrose agar (SDA) contains 1% chloramphenicol(Al-khalidi *et al.*, 2017, 2018) .The media were incubated at 32°C for ٧ days.

### 5-Phenotypic identification

After seven days of incubation, plate was observed for macroscopic characteristics such as colony diameter, exudates, colony reverse and the isolates were identified to the species level on the basis of microscopic characteristics including conidiophore, vesicle, metulae, phialides and conidia (Al-Khalidi *et al.*, 2018).

### 5.1. Preparation of lactophenol cotton blue stain

The stain lactophenol cotton blue was prepared according to the instructions of its manufacturer company, HIMEDIA, India fixed on its container, and components of this stain are: phenol 20 ml, lactic acid 20 ml, glycerol 40 ml and distilled water 20 ml. Reagents were mixed thoroughly. To each 100 ml of lactophenol 0.05 ml of cotton blue stain was added. It was stored at room temperature to be used for staining and microscopic identification of *Aspergillus* (Alkhalidi *et al.*, 2017, 2018).

### 5.2. Microscopic Examination

To get microscopic characteristics slides were stained with lactophenol cotton blue (Ellis *et al.*, 2007) by using adhesive tape production in which a tiny piece of transparent-adhesive tape was touched to the surface of the suspected colony, before adhered to the surface of a microscopic slide (Kirk *et al.*, 2008). Photographs were taken with digital microscopical camera.

First a morphological examination of the species was made at low magnification power of microscope and by naked eye after the in detail examination was done according to (Ellis *et al.*, 2007) by photographing the microscopic structures measuring the dimensions of the microscopic structures and using relevant literature.

## 6-PCR

### 6.1. DNA Extraction

DNA was extracted from *A. niger* by using Favor Prep™ total DNA mini Kit (FAVORGEN, Taiwan) according to the protocol stated by the Mini Kit manufacturer

### 6.2. dsDNA quantitation by Qubit 4.0

The assay is highly selective for double-stranded DNA (dsDNA) over RNA and is accurate for initial sample concentrations from 10 pg/µl to 100 ng/µl. The assay was performed at room temperature, and the signal was stable for 3 hours. Common contaminants such as salts, free nucleotides, solvents, detergents, or protein were well tolerated in the assay. The standard and short procedure.

### 6.3. Materials used for thermal Cycling Primers Selection and identification of *A.niger*, fumonisin and ochratoxin illustrated in Table (1)

### 6.4. Molecular confirmation of *A. niger* by NIGI and CMD1

This step was carried by adding 12.5 µl from one Taq (NEB®) master mix,3 µl of DNAsample,1 µl from each primer,7.5 µl from nuclease-free water (New England Biolabs, 2024).The reaction was done under the optimum PCR conditions shows in Table (1).

### 6.5. Molecular detection of Ochratoxin and Fumonisin

This step was carried by adding 12.5µl from one Taq (NEB®) master mix, 3 µl of DNA sample,1 µl from each primer,7.5 µl from nuclease-free water (New England Biolabs, 2024), the reaction was done under the optimum PCR conditions shows in Table (1) using applied biosystem thermal cycler.

### 6.6. Detection of PCR products by Gel Electrophoresis

**A. Preparation of 1X TAE Buffer**

Two hundred milliliter of TAE buffer 50x (0.08 M Tris, 0.08 M Acetic acid and 0.02 M EDTA) was diluted to 10X by taking 200 ml of 50X TAE and added to 800 ml of deionized distilled water (dd$H_2O$). This 10X buffer re-diluted to 1X (working concentration) by taking 100 ml and added to 900 ml of deionized distilled water (dd$H_2O$) (Alkhuwailidy & Alrufae, 2022).

**B. Preparation of Agarose Gel 2% and Visualization of PCR Products (Promega)**

Ten microliter of PCR product and DNA ladder had been loaded into the wells of gel. The voltage of power supply was fixed at 80V for 80 minutes. At the end of run, gel documentation with high resolution camera from thermos fisher had been used to capture image and analyze the bands (Alkhuwailidy & Alrufae, 2022).

**Table 1:** Details of the species-specific PCR primer pairs for *A. niger*, ochratoxin and Fumonisin

| Target | Name of the gene | | Sequence (5'- 3') | Denaturation | Annealing | Extension | Base pairs | Reference |
|---|---|---|---|---|---|---|---|---|
| ***A. niger*** | NIG1 | F | 5'-GATTTCGACAGCA TTT(CT/TC)CAGAA-3' | 94ºC for 30 sec. for 35x cycle | 48°C for 45 sec. for 35x cycle | 72°C for 45 sec. for 35x cycle | 290 bp | (Susca *et al.*, 2007) with modification |
| | | R | 5'-AAAGTCAATCA CAATCCAGCCC-3' | | | | | |
| ***A. niger*** | CMD 1 | F | 5'-GAG-TAC-AAG-GAG-GCC-TTC-TC-3' | 94ºC for 30 sec. for 35x cycle | 48°C for 45 sec. for 35x cycle | 72°C for 45 sec. for 35x cycle | 245 bp | (Abreu *et al.*, 2021) with modification |
| | | R | 5 ' - TCC-TT RGTGGTR-ATC-TGG-CCT- 3' | | | | | |
| Ochratoxin | PKS1 5KS | F | 5' -CAATG CCGTCC AACCGTATG-3' | 94ºC for 30 sec. for 35x cycle | 54°C for 45 sec. for 35x cycle | 72°C for 45 sec. for 35x cycle | 776-bp | (Kim *et al.*, 2014) with modification |
| | | R | 5' -CCTTCGCCTCG CCCGTAG-3' | | | | | |
| Fumonisin | Fum1.2 | F | 5' -CCATCGTGGGA TCTCAGAGATG-3' | 94ºC for 30 sec. for 35x cycle | 54°C for 45 sec. for 35x cycle | 72°C for 45 sec. for 35x cycle | 557-bp | (Kim *et al.*, 2014) with modification |
| | | R | 5' - CGCCAATGT CAA-GCATATGGTC-3' | | | | | |

The Initial denaturation for all primers was 94oC for 5 min. for 1 cycle.
The final extension of all primers was 72 °C for 7 min. for 1 cycle.

### 7- Detection of putative risk factors for infection with *Aspergillus among* Domestic Cats Breeders

A questions-based analysis was used for estimation of putative risk factors among breeders compared with the frequency of A. niger isolation from otomycosis including, age, sex, education level and economic status and occupation, swimming, using of antibiotics eardrops, trauma to the external ear canal, diabetes mellitus and absence of earwax.

### Statistical analysis:

Results were analyzed using Statistical Package for Social Scientist (SPSS version 18.0) (Al-Khalidi *et al.*, 2020; Najem *et al.*, 2020). Significant difference among means of the groups was determined by chi test, values were considered significant when $p<0.05$(Al-Ezzy, 2016, 2017), correlations were determined by correlation coefficient (Hameed & Al-Ezzy, 2024; Hameed *et al.*, 2024).

## RESULTS

### Fungal isolation and morphological identification on SDA for ear swabs of cat breeders

Table (2) shows the frequency of fungal species isolated from DCB according to morphological features on SDA as shown in Figure (1). The total number of isolated *A. niger* was 10.38% (11/106).

**Figure (1):** Macroscopic appearance of *A. niger* swabbed from external ear of DCB at 32ºC on SDA for 7 days.

**Table 2:** Fungal isolation status on SDA and morphological identification for ear swabs of DCB

| Source of sample | Isolation status on SDA | Total No. of swabs |
|---|---|---|
| Ear | Negative | 95 (89.62%) |
| | *A. niger* | 11(10.38%) |
| | Total | 106(100%) |

**Molecular identification of *A. niger* isolated from DCB by Conventional PCR**

All *A. niger* positive samples were subjected to confirmatory step by conventional PCR using species-specific PCR primer pairs for *A. niger* (NIG1) and (Cmd A). Length 245 bp and (290bp) guaranteed to be *A. niger* as shown in Figure (2) and (3), respectively.

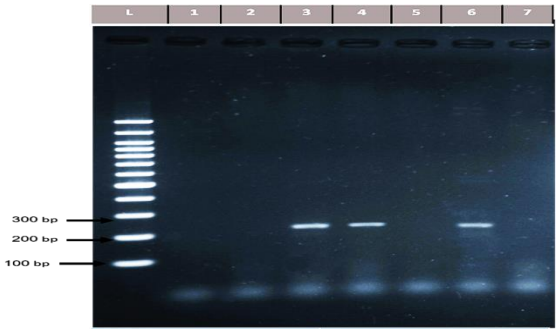


**Figure (2):** DNA products of *A. niger* isolated from ear of DCB generated through calmodulin gene (*cmdA*) primers, stained with ethidium bromide. M: Molecular marker (100bp), lanes 1,4,6,7,8 and 10 (245bp): positive samples for *A. niger* and lanes 2,3,5 and 9: negative samples.

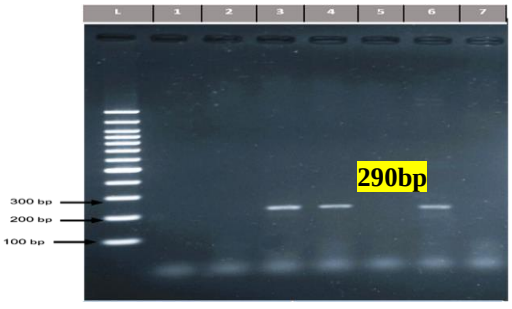


**Figure (3):** DNA products of *A. niger* isolated from ear of DCB generated through *NIG1* gene primers, stained with ethidium bromide. M: Molecular marker (100bp), lanes 3, 4 and 6 (290bp): positive samples for *A. niger* and lanes 1,2,5 and 7: negative samples.

**Detection of fumonisin and ochratoxin for *A. niger* isolated from Ear of DCB**

As shown in Table (3) and Figure (4), fumonisin was detected in 7/11(63.64%) of *A. niger* isolated from ear of DCB. Ochratoxin was not produced by any isolates of *A. niger*, 0/11, (0%) as shown in Table (3) and Figure (5).

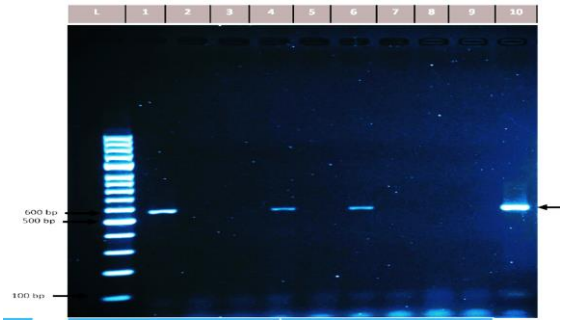


**Figure (4):** DNA products of fumonisin generated through *fum1* gene primers, stained with ethidium bromide. L: Molecular marker; lanes 4,6,10 (557bp): fumonisin positive samples and lanes 2,3,5,7,8 and 9: negative samples.

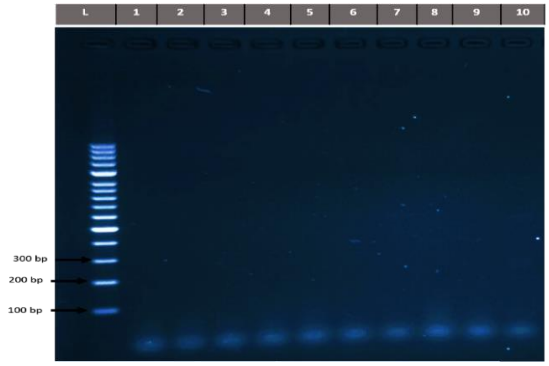


**Figure (5):** DNA products of ochratoxin generated through *pks* gene primers, stained with ethidium bromide. L: Molecular marker; lanes 1-10: No detection for ochratoxin DNA products (776pb).

**Table 3:** Frequency of PCR based detection for fumonisin and ochratoxin produced by *A. niger* isolated from ear of DCB

| Source of *A. niger* | External ear of DCB |
|---|---|
| **fumonisin** | 7/11(63.64%) |
| **ochratoxin** | 0/11 (0%) |

**Possible risk factors associated with *A. niger* Infection among DCB**

**1. Age susceptibility**

As shown in Table (4), swabs frequently taken from ear of DCB at the age group (41-48) years, 24/106(22.64%) in which *A. niger* was isolated from 3/106 (2.83%). No significant difference was reported between age groups infected with *A. niger*. Significant correlation was reported between age group and isolation of *A. niger*.

**2. Sex susceptibility**

A total of 56/106 (52.83%) of DCB were males versus 50/106 (47.16%) females. *A. niger* was isolated from 6/106 (5.66%) males versus 5/106 (4.71%). No significant correlation was recorded between sex and infection with *A. niger* (Table 4).

**Table 4:** Age, sex, education level and economic status and occupation as possible risk factors associated with *A. niger* infection among DCB

| Parameters | | *A. niger* Isolated From Ear Of Domestic Cats Breeders | R | P value |
|---|---|---|---|---|
| **Age group** | **9 -16** | 0(0%) | **0.195** | **0.045*** |
| | **17-24** | 2(1.88%) | | |
| | **25-32** | 1(0.94%) | | |
| | **33-40** | 1(0.94%) | | |
| | **41-48** | 3(2.83%) | | |
| | **49-56** | 2(1.88%) | | |
| | **57-64** | 2(1.88%) | | |
| | **Total** | **11(10.38%)** | | |
| **Sex** | **female** | 5(4.71%) | 0.12 | 0.905 |
| | **male** | 6(5.66%) | | |
| | **Total** | **11(10.38%)** | | |
| **Education level** | **Illiterate** | 0(0%) | 0.130 | 0.183 |
| | **Primary** | 1(0.94%) | | |
| | **secondary** | 1(0.94%) | | |
| | **Preparatory school** | 2(1.88%) | | |
| | **higher education** | 7(6.60%) | | |
| | **Total** | **11(10.38%)** | | |
| **Economic status** | **Middle** | 0(0%) | -0.164 | 0.093 |
| | **Good** | 5(4.71%) | | |
| | **Very good** | 6(5.66%) | | |
| | **Total** | **11(10.38%)** | | |
| **Occupation** | **Student** | 2(1.88%) | 0.015 | 0.882 |
| | **Public workers** | 3(2.83%) | | |
| | **Free works** | 3(2.83%) | | |
| | **Housewives** | 3(2.83%) | | |
| | **Total** | 11(10.37%) | | |

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

### 3. Education level

As shown in Table (4), 46/106 (44.33 %) of DCB had higher education level and 28 (26.41%) had secondary education level. *A. niger* was isolated mainly from those have higher education level, 7/106 (6.60%). *A. niger* was isolated equally from breeders with primary education and secondary education, 1/106 (0.94%). No significant correlation was reported between breeders' education levels and isolation of *A. niger*.

### 4. Economic status

As shown in Table (4), a total of 65/106 (61.32%) DCB had good economic status and 33/106 (31.13%) had very good economic status. *A. niger* was isolated mainly from breeders with very good economic status, 6/106 (5.66%) followed by those with good economic status, 5/106 (4.71%. No significant correlation was reported between economic status and isolation of *A. niger*.

### 5. Occupation

A total of 47/106 (44.33%) of breeders were housewives, 28/106 (26.41%) of DCB were public workers compared with 16 (15.09%) free workers and 15 (14.15%) were students (Table 4). *A. niger* was isolated equally from public workers, Free workers and housewives 3/106 (2.83%). No significant correlation (p value = 0.882) was reported between occupation and isolation of *A. niger*.

### 6. Swimming

As shown in Table (5), a total of 12/106 (11.32%) of DCB practiced swimming compared with 94/106 (88.67%) did not practice swimming. *A. niger* was isolated from breeders who practice swimming, 7/106 (6.60%), compared with 4/106 (3.77%) that they did not practice swimming. No significant correlation was reported between swimming and isolation of *A. niger*.

### 7. Using antibiotics eardrops

As shown in Table (5), A total of 12/106 (11.32%) of DCB using antibiotics eardrops compared with 94/106 (88.67%) did not use antibiotics eardrops. *A. niger* was isolated mainly from breeders did not use of antibiotics eardrops, 7/106 (6.60%) compared with 4/106 (3.77%) from those using antibiotics eardrops. Significant correlation (p value= 0.013) was reported between using of antibiotics eardrops and isolation of *A. niger*.

### 8. Trauma to the external ear canal

As shown in Table (5), a total of 100/106 (94.33%) of DCB whom did not suffer from trauma of the external ear canal compared with 6/106 (5.66%) exposed to trauma. *A. niger* was isolated mainly from breeders did not expose to trauma of the external ear canal, 10/106 (9.43%) compared with 1/106 (0.94%) from those exposed to trauma. No significant correlation (p value= 0.245) was reported between exposure to trauma and isolation of *A. niger*.

### 9. Diabetes mellitus

As shown in Table (5), a total of 98/106 (92.45%) of DCB did not suffer from diabetes mellitus compared with 8/106 (7.54%) with diabetes mellitus. *A. niger* was isolated mainly from breeders did not suffer from diabetes mellitus, 8/106 (7.54%) compared with 3/106 (2.83%) from those with diabetes mellitus. No significant correlation (p value = 0.881) was reported between diabetes mellitus and isolation of *A. niger*.

**Table 5:** Season, Absence Of Earwax, Swimming, Using Of Antibiotics Eardrops, Trauma to the External Ear Canal as Possible Risk Factor Associated With *A. niger* Infection Among Domestic Cats Breeders

| Parameters | | A. niger Isolated from ear of Domestic cat breeders Ear | R | P value |
|---|---|---|---|---|
| Season | Autumn | 3(2.83%) | 0.201 | 0.038* |
| | Winter | 8(7.54%) | | |
| | Total | **11(10.38%)** | | |
| Absence of earwax | Yes | 7(6.60%) | 0.220 | 0.024* |
| | No | 4(3.77%) | | |
| | Total | **11(10.38%)** | | |
| Swimming | Yes | 7(6.60%) | -0.044 | 0.653 |
| | No | 4(3.77%) | | |
| | Total | **11(10.38%)** | | |
| Using of Antibiotics eardrops | Yes | 4(3.77%) | 0.241 | 0.013** |
| | No | 7(6.60%) | | |
| | Total | 11(10.37%) | | |
| Trauma to the external ear canal | No | 10(9.43%) | -0.114 | 0.245 |
| | Yes | 1(0.94%) | | |
| | Total | 11(10.37%) | | |
| Diabetes Mellitus | No | 8(7.54%) | 0.015 | 0.881 |
| | Yes | 3(2.83%) | | |
| | Total | 11(10.37%) | | |

* Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

## DISCUSSION

The results of the current study showed that *A. niger* was successfully isolated from 11/106 (10.38%) samples. Current results come in contrary with (Haider *et al.*, 2022), who stated that *A. niger* represent 72.20% of fungal isolates in otitis externa while (Mistry & Pathak., 2021) and (Samorekar *et al.*, 2023), who concluded that *A. niger* was the most common agent for otomycosis in 62/103(60.19%), most likely as a result of its strong affinity for the external auditory canal and its capacity to generate a large number of conidia. This result was consistent with other research conducted by (Kumar *et al.*, 2017; Prasad *et al.*, 2014), who recorded that *A. niger* was the most prevalent pathogenic fungus. Current results considered higher than that reported by (Amigot *et al.*, 2003),who reported that *A. niger* was isolated from otitis externa (2.7%) and (Lee *et al.*, 2022) who stated that *Aspergillus* was isolated from 5.2% of the patients with chronic otitis externa. Current results come in line with (Sabz *et al.*, 2019) from Iran who reported that *A. niger* was isolated from (19%) of otomycosis. On the other hand, (Asoegwu *et al.*, 2023), who concluded that *A. niger* was isolated from 20.5% of otomycosis in Nigeria. While in other Nigerian study, *A.niger* otomycosis reported in 40% of patients (Yabo *et al.*, 2024)

Molecular identification confirmed that 11 isolated of *A. niger* by conventional PCR using species-specific PCR primer pairs for *A. niger* (NIG1) and (Cmd A). The primers utilized in this study were meticulously designed based on the calmodulin gene sequences of various *Aspergillus* strains, as outlined by (Susca *et al.*, 2007). This approach incorporated comprehensive phylogenetic and taxonomic analyses from preceding research (Susca, 2020). The calmodulin-based PCR method employed herein proved to be

highly specific for identifying *Aspergillus* species within the black *Aspergilli* group (Bufflier *et al.*, 2007; Susca *et al.*, 2007; Susca, 2020). This level of specificity contrasts sharply with the internal transcribed spacer (ITS) regions, which demonstrated a lower specificity due to variations in only three nucleotides among these species, as noted by Parenicova *et al.* (2000) and (Al-Hatmi, 2016; Parenicova *et al.*, 2000).

Previous studies showed varied results regarding isolation of *A. niger* from ear externa, consistence with current results such as (Praveena & Sowmya, 2023) from India stated that *A. niger* was isolated from 58.06% of patients presented with otomycosis while (Sarwestani *et al.*, 2019) from Iran showed that *A. niger* isolated from 37.59% of otomycosis and (Dekhil, 2018) from Iraq, 35.71% of total ear swab samples. On the other hand, (Mistry & Pathak., 2021), who reported that *A. niger* was isolated from 52.63% of otomycosis, while (Aggarwal & Jaiswal, 2019) demonstrated that *A. niger* constitutes only in 15.5% of total ear isolates, and (Zhang *et al.*, 2020) showed that *A. niger* isolated from 13.33% of external otomycosis. While other studies showed discrepancy with current results,(Kiakojuri *et al.*, 2015), who reported that *A. niger* was isolated from 55.36% of adolescent with otomycosis. While (Kiakojuri *et al.*, 2021), who recorded that *A. niger* was isolated from 8.86% of otomycosis ear swabs and in Nigeria, (Osazuwa *et al.*, 2011), who concluded that A. niger was isolated from 9.2% of otomycosis cases. (Osazuwa *et al.*, 2011) stated that *A. niger* was likely a secondary invader rather than a primary pathogen, as most patients had undergone antibiotic treatment prior to the isolation of *A. niger* from their ears. The prevalence of *A. niger* in the outer ear may be attributed to its resistance to the fungistatic properties of ear wax (cerumen).

On the other hand, (Mohammed *et al.*, 2020) reported that *A. niger* was isolated from 43% of otomycosis in samarra city, Iraq . While, (Mahmoudabadi *et al.*, 2009) from Iran, showed that *A. niger* isolated from 47.1% of samples. In Iran, (Saki *et al.*, 2013) stated that *A. niger* isolated from 67.2% of otomycosis. These result considered very high compared with that reported by (Kiakojuri *et al.*, 2021) demonstrated that 8.86% of isolation rate was *A. niger* based on molecular identification of fungi from patients with otomycosis and (Sabz *et al.*, 2019) recorded that 19% of isolation rate was *A. niger* of fungi from patients with otomycosis according to real time PCR based.

A. niger is typically considered a non-pathogenic fungus that is prevalent in various environments. Humans encounter its spores on a daily basis without manifesting any symptoms of disease. However, there are rare instances where *A. niger* can act as an opportunistic pathogen, colonizing the human body. Such occurrences are predominantly observed in individuals with a history of serious illnesses or those undergoing immunosuppressive therapies (Plascencia-Jatomea *et al.*, 2014).

Otomycosis leads to local inflammation and mycelial growth on cerumen on the skin of the external ear canal. Although relatively common in tropical climates, otomycosis is not considered a severe condition and can be effectively treated with topical antifungal ointments (Adhavan, 2020). Self-cleaning of the ears, which can cause mechanical damage to the skin barrier, is a significant factor contributing to the occurrence of otomycosis (Vivas & Hanson, 2023).

Fumonisin are also a group of toxic secondary metabolites that originate from black *Aspergillus* species and thus can be viewed as a possible source of mycotoxin contamination. The results of current study appear to be the first in clinical veterinary and human aspect regarding detection of FUM1 gene for fumonisin. In current study fumonisin *FUM1* gene was detected in 63.64% of *A. niger* isolated from ear swabs of DCB.

In current study, ochratoxin was not detected in all *A. niger* positive cases which come in contrary with that reported by (Mohammed; *et al.*, 2020), who stated that out of all the examined isolates, 60% of the findings of the PCR technique indicated the presence of the

PKS gene, which is the gene responsible for manufacturing ochratoxin A in *A. niger*. The findings of this study go beyond those of study (Fairooz & Sameer, 2016), who discovered that 35% of the isolates of the *A. niger* fungus carried the PKS gene, which is responsible for the production of ochratoxin A. In a separate study, (Almusawy 2015), reported that 66.6% of the *A. niger* isolates isolated from the blood and urine of patients suffering from kidney failure, and expressed ochratoxin A gene.

Results of the current study showed no significant association between age and *A. niger* infection among DCB, with higher frequency at the age group (41-48 years), while there is no infection in younger age groups (9-16 years). These results come in contrast with (Praveena & Sowmya, 2023), who concluded that 9.68% of patients were at (41-50years). Current results come closely to (Mistry & Pathak., 2021) from India who stated that 23.68% of patients with otomycosis at the age group (31-40years) and come in agreement with (Kiakojori *et al.*, 2018; Roohi *et al.*, 2023) from Iran and that reported by (Asoegwu *et al.*, 2023), who reported that *A. niger* otomycosis do not correlated with age although, the tendency was higher at younger age (1-10years), with high frequency of isolation (25%).

Similarly, (Onotai & Osuji, 2016) noted that the peak prevalence of otomycosis was between 1 and 10 years (30.7%) and 21 and 30 years (26.2%). In other research, by (Yabo *et al.*, 2024), they reported that the preponderance age was (11-20years).While the age range of 20–40 years old had the highest occurrence (Abdelazeem *et al.*, 2015; Aggarwal & Jaiswal, 2019; Fasunla *et al.*, 2008; Gupta & Mahajan, 2015). The age groups with the lowest prevalence were those under 10 and those over 60 (Prasad *et al.*, 2014). Current results come in contrary with (Iwewe *et al.*, 2023), who stated that 35% of otomycosis detected at the age group (20-30 years ), only 1% of patients at the age group (40-50years).

Current results were consistence with the study of (Wang *et al.*, 2022), they reported that age groups 41-60 and 61-80 years old were the most frequent regarding *A. niger* isolation. While (Lotfali *et al.*, 2022) stated that high prevalence of positive *A. niger* and *A. fumigatous* otomycosis at the age group (50-59 years) were 26.2%. While (Kazemi *et al.*, 2015) showed that most cases were found in patients aged between 21-40 years. A high prevalence of *A. niger* in these age groups may be associated with occupational activity, as a significant number of individuals in these age groups hold jobs involving agriculture, horticulture, harvesting, farm work, and seasonal construction work that often take place in small cities and dusty environment (Kazemi *et al.*, 2015).

Young individuals were more vulnerable, which may be the reason why middle-aged persons were more affected in both the current study and the ones mentioned previously. Owing to their jobs, travel, and other circumstances to fungi (Jyoti *et al.*, 2019; Samorekar *et al.*, 2023).

The current study revealed no significant correlation between *A. niger* and sex, with slightly higher prevalence in males, which come in agreement with (Haider *et al.*, 2022) from Bangladesh, who reported that 56% of otomycotic patients were males versus (44%) females. On the other hand, (Yabo *et al.*, 2024) stated that no significant correlation between sex and *A. niger* otomycosis and reported that 52% of *A. niger* otomycosis were female compared with 48% were males. Similar result was recorded by (Praveena & Sowmya, 2023), who demonstrated that 63.29% of patients with otomycosis were males versus (36.71%) were females. On the other hand, (Lotfali *et al.*, 2022) concluded that 55.8% of patients presented with otomycosis with *A. niger* and *A. fumigatous* positive culture were males compared with 44.2% were females. Another study by (Kiakojori *et al.*, 2018) from Iran with tendency to infection for females at (33.78%) compared with (26.31%) of males Current results come in contrary with (Dekhil, 2018) from Iraq and (Roohi *et al.*, 2023) from Iran who recorded

that the majority of patients (65% and 56.4%) with otomycosis, respectively were females.

In contrary with that reported by (Iwewe *et al.*, 2023) from Nigeria, stated that 62% of patients attended to ear, nose, and throat clinics with otomycosis were females. Current results come in line with (Ho, T., *et al.*, 2006), who reported that 56% of the total population presenting with otomycosis were males. Previous study of (Wang *et al.*, 2022) found that *A. niger* was more prevalent in males, while, (Aneja *et al.*, 2010; Dekhil, 2018), who found *A. niger* was more prevalent in females. Nemati *et al.* (2014) recommended that head scarf was a significant reason for otomycosis in women. On the other hand, head scarf may prevent ears from fungal spores getting into the ear canal (Gharaghani *et al.*, 2015). Further, Barati *et al.* (2011) stated that wearing head scarf was not a possible predisposing factor for fungal ear infection. This discrepancy in sex among different studies may be due to variations in associating risk factors and climatic conditions.

This study failed to establish any correlation between the education level and infection with *A. niger*. Education level was used as a covariate in order to assess if it had any effect on the vulnerability of human beings to *A. niger* otomycosis. In current study *A. niger* was isolated mainly from those have higher education level (6.60%), primary education and secondary education (0.94%) with this consideration, however, analysis by means of correlation coefficients proved negative. These results come in contrary with (Haider *et al.*, 2022), who found that 33% of otitis externa including those with otomycotic patients had primary education level compared with those for higher education (13.5%).This indicates that there might be other factors that are more determinant in the occurrence of otomycosis due to *A. niger*.

Current study reported no correlation between economic status of DCB and A. niger otomycosis, which come in contrary with that reported in Iraq by (Jameel & Al-Ezzy, 2017), they stated that significant inverse correlation between *A. niger* otomycosis and family economy. (Deshmukh *et al.*, 2014) found that 64% of *Aspergillus* positive otomycosis were of low economic level at rural area in Maharashtra state, India. On the other hand, (Krishna *et al.*, 2022) stated that 30% of otomycosis positive cases were at lower economic status and 22-28% with middle level. Present study come in contrary with that reported in Taiz, Yemen by (Raweh *et al.*, 2020), who observed that *A. niger* otomycosis was prevalent in poor population with low economy.

Current investigation reported high detection rate of *A. niger* otomycosis in winter compared with autumn which come in accordance with (Aneja *et al.*, 2010; Haider *et al.*, 2022; Mahmoudabadi *et al.*, 2010). The results of this study come in contrast with (Barati *et al.*, 2011), who observed that the incidence of *A. niger* otomycosis was highest in the autumn followed by the summer, winter and spring. While in contrast with current results, it was also observed that infection was higher in summer as concluded by (Gharaghani *et al.*, 2015).

Although, *A. niger* was isolated from external ear canal in winter, present study reported no significant correlation between season and *A. niger* otomycosis which come in agreement with (Asoegwu *et al.*, 2023; Jameel & Al-Ezzy, 2017), who stated that the infection was frequent in runny season which proved by suitable temperature and humidity.

A significant contributing cause to the rising incidence of ear fungal infections is the air's high concentration of suspended dust particles. Fungi, which are prevalent on decomposing plant waste, can be taken away by water vapor during the rainy season when they are blown in the wind together with soil particles. This is in good agreement with the increased incidence of fungal infections during the wet season (Fasunla *et al.*, 2008).

A typical biological process in humans and many other mammals is the production of cerumen or earwax. Cerumen acts as a barrier to keep out water, foreign objects, insects, and

other arthropods while also hydrating and shielding the skin of the external auditory canal from infection. Normally, normal jaw movement causes cerumen to naturally evacuate from the ear canal (Michaudet & Malaty, 2018).

The findings of this study indicate a significant correlation between the absence of ear wax and *A. niger* otomycosis. Current result come in accordance with (Prasanna *et al.*, 2014), who found that the neutralization or alkalization of the canal and the lack of cerumen's protective covering undermine the body's natural defense against bacterial or fungal contamination.

Ear wax, or cerumen, plays a crucial protective role by lubricating the ear canal and trapping foreign materials. Its acidic pH provides an inhospitable environment for bacterial and fungal growth, thereby offering antibacterial and antifungal properties. Cerumen contains chemical compounds that help in cleaning, lubricating, and protecting the ear canal from microbial invasions (Pereira *et al.*, 2024). The lack of cerumen likely compromises these protective functions, increasing susceptibility to infections such as otomycosis caused by *A. niger* (Agrawal & Deshmukh, 2021)

There was non-significant relationship between *A. niger* and swimming and individuals that swim were more exposed to *A. niger*. These results come in agreement with (Krishna *et al.*, 2022), who recorded that among 44% of otomycosis positive patients, swimming represent the vulnerable risk factor. On the other hand, (Aneja *et al.*, 2010; Gharaghani *et al.*, 2015) demonstrated that swimming was risk factor for fungal ear infections, specifically *A. niger*. One of the most important factors for otomycosis is long-term exposure to moisture: the prevalence of infection was reported to be five times higher in swimmers than in non-swimmer (Khan & Jain, 2019; Vennewald & Klemm, 2010). As stated by numerous studies, the increased frequency of otomycosis might be attributed to excessive humidity, wetness, bathing, swimming, and personal cleanliness (Haider *et al.*, 2022; Kazemi *et al.*, 2015; Khan & Jain, 2019).

The results showed that the use of antibiotics eardrops significantly correlated with *A. niger* infection which come in agreement with (Pereira *et al.*, 2024) from India who stated that 47% of patients with positive otomycosis using ototopical antibiotics drops. On the other hand, (Dekhil, 2018) from Iraq and (Agarwal & Devi, 2017) who recorded that the used antibiotic ear drops represent the main risk factor which was reported in 59.4% of otomycosis patients. Similar results were reported by (Aneja *et al.*, 2010) from India and (Sangaré *et al.*, 2021) from Burkina Faso. While, (Lotfali *et al.*, 2022) from Iran reported that 56.5% of *A. niger* positive otomycosis patients using antibiotic ear drops. Also, (Deshmukh *et al.*, 2014) in Maharashtra, India, found that 67% of *A. niger* positive patients with otomycosis utilize ear antibiotic drops.

The impact of topical antibiotic ear drops on the development of ear fungal infections was examined in a study by Jackman *et al.* (Jackman *et al.*, 2005). Their findings suggested that ofloxacin may have two potential roles in the development of otomycosis: first, because it is bactericidal to the majority of bacteria in the external auditory meatus, fungal proliferation may occur due to a lack of competition for bacterial growth; and second, because, unlike other topical antibiotic ear drops, which typically have a pH of 3-4, ofloxacin ear drops have a pH of 7, which is a more neutral solution and will not acidify the external auditory canal skin, creating an environment that is more favorable for fungal growth and proliferation.

Current study reported no significant correlation between diabetes mellitus and *A. niger* otomycosis and only (2.83%) come with positive culture which come in agreement with that reported in India by (Deshmukh *et al.*, 2014). In a study done by (Hydri *et al.*, 2017), who reported that only 9.39% of diabetic patients presented with *A. niger* otomycosis which indicate multifactorial induce *A. niger*

infections. In diabetic patients, cerumen usually with high PH and low lysozyme activity which impaired local ear immunity and predispose to otomycosis.

A total of 47/106 (44.33%) of DCB were housewives, 28/106 (26.41%) were public workers compared with 16/106 (15.09%) were free workers and 15/106 (14.15%) were students. *A. niger* was isolated equally from public workers, free workers and housewives 3/106 (2.83%). No significant correlation (p value= 0.882) was reported between occupation and isolation of *A. niger* which come in agreement with (Yabo *et al.*, 2024), who observed that 79% of patients with otomycosis were students, 4% of housewives, and 2% of free workers. Current results come in accordance with that reported by (Asoegwu *et al.*, 2023), who stated that the male and female occupations for patients with otomycosis did not differ from one another. The adults were either professionals, business owners, civil servants, or retirees, while the youngsters were either students or pupils. There were not many homemakers (Fasunla *et al.*, 2008). Therefore, in this study, otomycosis was not linked to any employment.

A total of 100/106 (94.33%) of DCB did not suffering from trauma of the external ear canal compared with 6/106 (5.66%) those exposed to trauma. *A. niger* was isolated mainly from breeders did not expose to trauma of the external ear canal, 10/106 (9.43%) compared with 1/106 (0.94%) from those exposed to trauma. No significant correlation (p value= 0.245) was reported between exposure to trauma and isolation of A. *niger*.

Chronic use of wooden sticks and metal wax pickers were revealed to be the predisposing factor causing injury to the ear canal according to (Jahan *et al.*, 2019) and (Ahuja *et al.*, 2017).

The present study showed no significance difference regarding to trauma of external ear canal. Previous study conducted by (Sarwestani *et al.*, 2019) concluded that trauma represent an important risk factor predisposing for fungal ear infections.

## CONCLUSIONS

*A. niger* ear infection represents significant problem among DCB. Using of NIG1 and Cmd A has equal significance in molecular diagnosis of *A. niger*. Age group, season, absence of earwax, using of antibiotics eardrops have serious effect on isolation of *A. niger* form ear of DCB. Fumonisin appears to be the major mycotoxin produced from *A. niger* otomycosis.

### Perspectives for Future Research

Study the molecular regulation of fumonisin and ochratoxin in otomycosis among cat breeders and evaluation of mycotoxins in innate immunity markers and study the phylogenetic tree of *A. niger* isolates from cats and breeders.

### Author Contributions

Authors disclosed that they were equally contributed in planning, methodology, data collection and analysis, and writing of manuscript.

### Funding Disclosure

Authors disclosed that they share the total cost of current research project.

### Conflict of Interest Disclosure

Authors disclosed that there was no conflict of interest.

### Artificial Intelligence Disclosure

Authors disclosed that AI was not used entirely in current research.

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# الكشف الجزيئي عن جينات NIG1 و CMDA لفطريات الرشاشيات نيجر *ASPERGILLUS NIGER* وجينات السموم الفطرية FUM1 و PKS من داء فطريات الأذن بين مربي القطط المنزلية مع التركيز بشكل خاص على عوامل الخطر في محافظة ديالى، العراق

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فطريات الرشاشيات هي فطريات انتهازية تعد سببًا رئيسيًا لداء الفطريات الأذنية في الانسان. تهدف الدراسه لعزل الرشاشيات النيجيرية من الأذن الخارجية لمربي القطط المنزلية والتوصيف الجزيئي للرشاشيات النيجيرية بواسطة تفاعل البوليميراز المتسلسل التقليدي ، الكشف عن عوامل الخطر المفترضة للإصابة بالرشاشيات، والكشف عن الفومونيسين والأوكراتوكسين بواسطة تفاعل البوليميراز المتسلسل. تم أخذ مائة وستة مسحات أذن خارجية لمربي القطط التي حضرت إلى العيادات البيطرية الخاصة وتم زراعتها على أجار سابورود ديكستروز. تم التعرف على النمو الفطري على أنه الرشاشيات النيجيرية وفقًا للخصائص الزرعيه. خضعت جميع العينات الإيجابية للرشاشيات النيجيرية لخطوة تأكيدية عن طريق تفاعل البوليميراز المتسلسل باستخدام البادئات الخاصةبالرشاشيات النيجريه **(NIG1)** و **.(Cmd A)**تم التأكد من إنتاج الفومونيسين والأوكراتوكسين بواسطة الرشاشيات النيجيرية بعن طريق تفاعل البوليميراز المتسلسل باستخدام بادئات محددة. تم عزل الرشاشيات النيجيرية من ١٠,٣٨٪ من مسحات الأذن لمربي القطط.

وجد ارتباط كبير بين الفئة العمرية، والموسم، وغياب شمع الأذن، واستخدام قطرات المضادات الحيوية لالأذن وعزل الرشاشيات النيجيرية . لم يتم تسجيل أي ارتباط معنوي بين الجنس، ومستويات التعليم، والحالة الاقتصادية، والسباحة، والتعرض للصدمات، ومرض السكري من النوع الثاني والمهنة وعزل الرشاشيات النيجيرية.تم الكشف عن فومونيسين **(FUM1)** في (١١/٧) ٦٣,٦٤٪ من الرشاشيات النيجيرية المعزولة من أذن مربي القطط المنزلية .لم يتم إنتاج أوكراتوكسين **(PKS)** في أي من عزلات الرشاشيات النيجيرية ، ٠٪ (١١/٠).إن استخدام **NIG1** و **Cmd A** له أهمية متساوية في التشخيص الجزيئي للرشاشيات النيجيرية

الفئة العمرية، والموسم، وغياب شمع الأذن، واستخدام قطرات المضادات الحيوية لالأذن لها تأثير خطير على اصابه الاذن بالرشاشيات النيجيرية بين مربي القطط المنزلية .

إن استخدام البوادئ **NIG1** و **Cmd A** له أهمية متساوية في التشخيص الجزيئي للرشاشيات النيجيريه. الفئة العمرية، والموسم، وغياب شمع الأذن، واستخدام قطرات المضادات الحيوية لالأذن لها تأثير خطير على اصابه الاذن بالرشاشيات النيجيريه بين مربي القطط. الفومونيسين هو السم الفطري الرئيسي الذي ينتج من االرشاشيات النيجيريه المسببه لفطار الأذن.

تمثل عدوى الاذن بالرشاشيات النيجيريه مشكلة كبيرة بين مربي القطط

***الكلمات المفتاحية:*** الرشاشيات النيجيرية، فطار الاذن ، مربي القطط المنزلية، فومونيسين، أوكراتوكسين، عوامل الخطر