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Phenotypic Identification and Molecular Characterization of Aspergillus Niger, Fumonisin and Ochratoxin Genes from Otomycosis in Domestic Cats with Special Emphasis to Underlying Risk Factors

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Abstract

The current study aims to isolation of Aspergillus niger from external ear of cats, molecular characterization of A. niger, fumonisin and ochratoxin by conventional PCR and detection of risk factors. A total of 106 external ear swabs were taken from domestic cats and cultured on Sabouraud dextrose agar. Positive fungal growth was identified by conventional PCR using species-specific PCR primer pairs for A. niger (NIG1) and (Cmd A)—production of fumonisin and ochratoxin confirmed by PCR using Fum 1 and PKS specific primers. The total of (20.75 %) swabs were A.niger positive by culture and PCR using (NIG1) and (Cmd A) primers. Cats with age (2-6) months were frequently infected followed by those with (12- 16) months. Males represent (62.26%) while female represent (37.73%). A total of (15.09%) males were infected with *A.niger* versus (5.66%) for females . Females appear to be at risk of getting A. niger infection at (1.813) time than males. No significant correlation was reported between age, sex, body weight; breed; hair length; ear cleaning interval; bathing frequency; season and infection with A. niger. Fumonisin was detected in (41.17%) of A. niger isolates while ochratoxin was not detected.

Keywords: Aspergillus niger, Fumonisin, Ochratoxin, otomycosis, cat

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Introduction

Otomycosis is a condition of the external ear canal in cats, caused by a fungal infection. In order for the condition to occur, there usually needs to be a change in the external ear canal environment or damage to the skin within the canal(Mokhtar, 2022). Because fungi thrive in warm, moist environments, the presence of excessive wax within the ear canal provides an excellent incubator for the fungi(Goodale *et al.*, 2016). Persians and other breeds with long hair and/or hairy ears are more susceptible to ear infections, especially in hot, humid weather(Harvey, 2016). Although otomycosis is found more often in dogs, it can still be quite prevalent in cats. The most common cause of otomycosis in cats is when the ear canal is damaged from excessive cleaning or scratching due to an ear mite infestation(Brame; & Christine, 2021). Without any ear injury, a healthy cat is relatively resistant to fungal infections (Kalundia, 2024). However, in some cases, when there is an overabundance of a particular type of fungi in the environment, a healthy ear can still become infected(Hernandez & Martinez., 2018). It is incredibly important to remember that fungal infections of other parts of the body are often indicative of an underlying disease process. So, if the veterinarian does not find any predisposing cause for the ear infection, he may need to run additional tests to look for possible systemic health issues(Eissa, 2024).

There are over 180 species of aspergillus common in the environment and cause disease in cats and dogs if has a compromised immune system or one exposed to a very large amount of fungus (Elad & Segal, 2018). In most cases, the fungal infection mainly confined to the sinus/or bit region in cats may spread on the body in severe cases. Two types of aspergilli can occur in cats: (nasal aspergillosis or upper respiratory tract aspergillosis) and (disseminated aspergillosis, or systemic aspergillosis). Nasal aspergillosis is the most common form and zoonotic form(Ugochukwu *et al.*, 2022). Infection occurs by breath in microscopic Aspergillus spores and grows in the nose, nasal cavity and sinuses(Fawsitt *et al.*, 2023). The disseminated aspergillosis may be due to a poorer immune system (Hartmann *et al.*, 2013)

Current study aims to isolation of A.niger from external ear of cats, Molecular characterization of A. niger by Conventional PCR , Detection of putative risk factors for infection with A.niger, detection of fumonisin and ochratoxin by PCR.

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Methods:

Ethical Consideration:

The current clinical study was performed in accordance with the guides for the care and use of laboratory animals and confirmed by the ethics committee at pathology department, college of veterinary medicine, University of Diyala Iraq. The approval No.CVM-DP-25 /202.

Area of the study:

Current study was achieved in veterinary clinics at Diyala governorate(Al-Ezzy, 2016a; Hassan *et al.*, 2020) and its capital city(Hameed & Al-Ezzy, 2019; Fajer *et al.*, 2023b) ,Baqubah northeast Baghdad, Iraq(Al-Ezzy *et al.*, 2015; Fajer *et al.*, 2023a).

Collection of Samples

The study included 106 domestic cats suffered from otomycosis. Age range (2-36 months) with mean (10.2170 ± 0.73months). The minimum body weight was 300 gram while maximum weight was 6000 grams, and the Mean± SE was 1.57868 ± 0.114815 gram. Swabs from external ear were taken and transfer to mycology lab. at the college of veterinary medicine, University of Diyala. Swabs were cultured on Sabouraud dextrose agar medium .Positive culture was reported according to phenotypic characters according to the references(Al-khalidi *et al.*, 2017).

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, components of this stain are: Phenol 20ml, Lactic acid 20ml, Glycerol 40ml and Distilled water 20ml..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(Al-khalidi et al., 2017, 2018).

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

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microscopic structures and using relevant literature . After culturing of ear swabs on SDA medium ,each culture positive, slide was prepared and stained by LPCB for co-nidia or hyphae indicated positive fungi in-fection (Mokhtar, 2022)

PCR Based Molecular Methods:

DNA Extraction

DNA was extracted from A. niger by using FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit(FAVORGEN, Taiwan) according to the protocol stated by the mini kit manufacturer (Favorgen, 2024)

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 is performed at room temperature, and the signal is stable for 3 hours. Common contaminants such as salts, free nucleotides, solvents, detergents, or protein are well tolerated in the assay. The standard and short procedure. Primers Selection and identification of A. niger, fumonisin and ochratoxin illustrated in table (1).

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

target	Name of the gene	Sequence (5'- 3')		Initial denaturation	Denaturation	Annealing	Extension	Final extension	Base pairs	Reference
A.niger	NIG1	F	5'-GATTTCGACAG-CATTT(CT/TC)CAGAA-3'	94c for 5 min.(for 1 cycle)	94c for 30 Sec..(for 35x cycle)	48°C for 45s .(for 35x cycle)	72 °C for 45 sec. (for 35x cycle)	72 °C for 7 Min .(for 1 cycle)	290bp	(Susca *et al.*, 2007)with modification
		R	5'-AAAGTCAATCACAAT CCAGCCC-3'							
ochratoxin	PKS15 KS	F	PKS15KS-f (5 -CAATGCCGTCCAAC-CGTATG-3)	94c for 5 min.(for 1 cycle)	94c for 30 Sec..(for 35x cycle)	54°C for 45s .(for 35x cycle)	72 °C for 45 sec. (for 35x cycle)	72 °C for 7 Min .(for 1 cycle)	776-bp	(Kim *et al.*, 2014) with modification
		R	PKS15KS-r (5 -CCTTCGCCT CGCCCGTAG-3)							
Fumonisin	Fum1	F	fum1.2F(5 -CCATCGTGGGA TCTCAGAGATG-3)	94c for 5 min.(for 1 cycle)	94c for 30 Sec..(for 38x cycle)	54°C for 45s .(for 38x cycle)	72 °C for 45 sec. (for 38x cycle)	72 °C for 7 Min .(for 1 cycle)	557-bp	(Kim et al., 2014) with modification
		R	fum1.2R (5 – CGCCAATGT CAA-GCATATGGTC-3)							

Molecular Detection of A. niger

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

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Molecular detection of Ochratoxin and Fumonisin

This step was Carried by adding 12.5µL from oneTaq(NEB ®) master mix,3 µL of DNAsample,1µL from one each primer,7.5 µL from Nuclease-Free water(New England Biolabs, 2024), the reaction done under the optimum PCR conditions shows in Table(1)

Protocol of Gel Electrophoresis

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 (ddH_2O). This 10X buffer re-diluted to 1X (working concentration) by taking 100 ml and added to 900 ml of deionized distilled water(ddH_2O)(Alkhuwailidy & Alrufae, 2022).

Preparation of Agarose Gel 2% and Loading of Samples into Gel

Ten microliter of PCR product and DNA ladder have 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 have been used to capture image and analyze the bands(Alkhuwailidy & Alrufae, 2022).

Statistical Analysis:

Data were analyzed using Statistical Package for Social Scientist (SPSS version 18.0)(Al-Ezzy *et al.*, 2016; Al-Khalidi *et al.*, 2020). Significant difference among means of the groups was determined by chi test(Al-Ezzy, 2015; Hameed *et al.*, 2020), values were considered significant when $p<0.05$(Al-Ezzy, 2016b, 2017; Al-Ezzy *et al.*, 2017).Correlations were determined by correlation coefficient (Hameed & Al-Ezzy, 2024; Hameed *et al.*, 2024).

Results

A s shown in figure (1) and table (2), the identification of *A.niger* isolated from domestic cat according to morphological features on SDA . The total number of A.*niger* isolated from ear of domestic Cat was 22/106 , (20.75 %) .

Molecular Identification of A.niger Isolated From domestic cat 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 guaranteed to be A. niger as shown in figure (2) and (3)

Table (2): Morphological Identification of *Fungal Species* Isolated from Ear of Cat

Source of sample for cat	Isolation status on SDA	Total No.
Ear	No growth	84 (79.24%)
	A. niger	22(20.75 %)
	Total	106(100%)

Figure (1): Macroscopic appearance of A.niger swabbed from external

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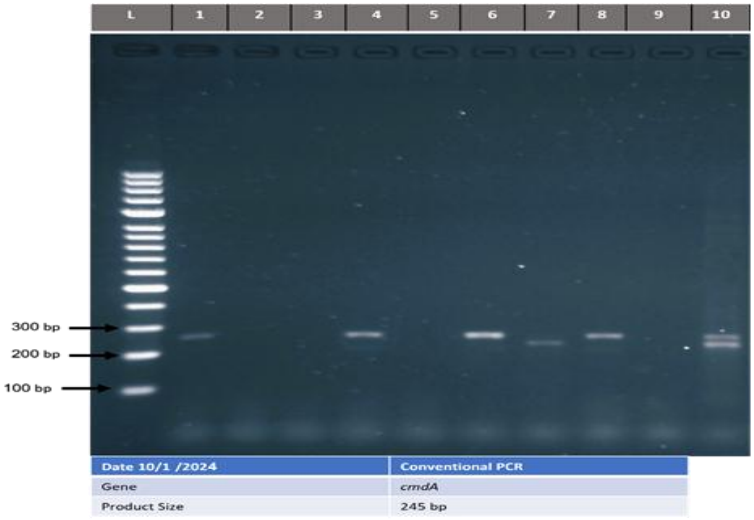

Figure (3): DNA products of *A. niger* generated through calmodulin gene (cmdA) primers, stained with Ethidium bromide. M : Molecular marker (100bp); lanes 1-10 (245bp) , *A.niger*

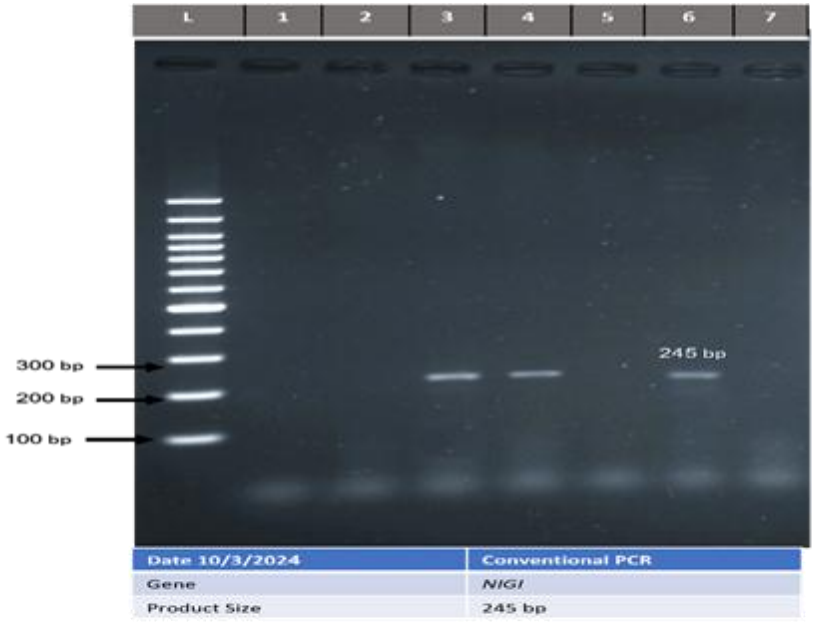

Figure (2): DNA products of *A. niger* generated through (NiGi) primers, stained with Ethidium bromide. M : Molecular marker (100bp); lanes 1-7 (245bp) , *A.niger*

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Possible Risk Factor Associated with *A. niger* Infection among Domestic cats

Age as a possible Risk Factor As shown in table (3), cats with age (2-6) months were frequently infected with A.niger 8/106,(7.54%) followed by those with (12- 16) months 7/106,(6.60%).A.niger was not recover from old age , (17-21)months, (27-31))months . No significant difference nor correlation were reported between age of domestic cat and infection with A.niger .

Sex as a possible Risk Factor

Current study revealed that 66/106 of Domestic cat were males (62.26%) while female represent 40/106 (37.73%). Males were infected with A.niger isolated from ear , 16/106 (15.09%)versus 6/106(5.66%) for females as shown in table (4). No significant difference nor correlation were reported between sex of domestic cat and infection with A.niger. Females appear to be at risk of getting A. niger infection at (1.813) time than males, as shown in table (3)

Breed as a possible Risk Factor

As shown in table (3), Persian cat breed was the most common attendance to veterinary clinics, 33/106, (31.13%) followed by Himalayan, 31/106, (29.24%). the least one was Sphynx, 3/106, (2.83%).A.niger was isolated primarily from ear of Himalayan domestic cat 9/106 ,(8.49%),followed by Persian, 7/106,(6.60%) and Shirazi breed, 4/106,(3.77%). A.niger was isolated in minimum frequency from Calico and Sphynx breeds ,1 /106,(0.94%). No significant difference nor correlation were reported between breed of domestic cat and infection with A.niger.

Table (3): Age as A Possible Risk Factor Associated with *A. niger* Infection among Domestic cats

Parameters	Age group (months)	Fungal growth from ear of Domestic cat			χ2	P value	R	P value
		no growth	A. niger	Total				
Age	2-6	29(27.35%)	8(7.54%)	37(34.90%)	7.476	0.876	0.019	0.844
	7-11	22(20.75%)	4(3.77%)	26(24.52%)				
	12- 16	23(21.69%)	7(6.60%)	30(28.30%)				
	17-21	1(0.94%)	0(0%)	1(0.94%)				

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	22-26	6(5.66%)	2(1.88%)	8(7.54%)				
	27-31	0(0%)	0(0%)	0(0%)				
	32-36	3(2.83%)	1(0.94%)	4(3.77%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				
Sex	Female	34(32.07%)	6(5.66%)	40(37.73%)	1.294	0.255	0.110	0.260
	male	50(47.16%)	16(15.09%)	66(62.26%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				
	Risk Estimate (Females Vs Males) = 1.813		95% Confidence interval =0.644-5.102					
Breed	Persian	26(24.52%)	7(6.60%)	33(31.13%)	4.364	0.359	0.063	0.518
	Himalayan	22(20.75%)	9(8.49%)	31(29.24%)				
	Shirazi	16(15.09%)	4(3.77%)	20(18.86%)				
	Calico	18(16.98%)	1(0.94%)	19(17.92%)				
	Sphynx	2(1.88%)	1(0.94%)	3(2.83%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				

Body weight as a possible Risk Factor

As shown in table (4) , A.niger was isolated primarily from ear of domestic cat with body weight (3001250— gram), 12/106,(11.32%) followed by those with body weight (1251-2051gram),6/10,6 (5.66%) followed by (3654-4454 gram)2/106 **,(1.88%)**.Minimum isolation of A.niger was reported among those with body weight (2052-2852 gram),(2853-3653 gram),1 /106 ,(0.94%). No significant difference nor correlation were reported between body weight of domestic cat and infection with A.niger.

Hair length as a possible Risk Factor

As shown in table (4), cat with Hair length was the most common attendance to veterinary clinics, 95/106, (89.62%) followed by short hair , 11/106 ,(10.37%). A.niger was isolated primarily from those with long hair , 18/106,(16.98%) compared with 4 /106,(3.77%) from ear of short hair cats. No significant difference nor correlation were reported between hair length of domestic cat and infection with A.niger .

Ear cleaning interval as a possible Risk Factor

As shown in table (4), cat with ear cleaning interval of more than two weeks represent 77/106, (72.64%) compared with 29/106, (27.35%) for those with cleaning interval of less than two weeks. A. niger was isolated primarily from those with ear cleaning interval of more than two weeks, 18/106, (16.98%) compared with 4 /106, (3.77%) from ear of those with ear cleaning interval of less than two weeks. No significant difference nor correlation were No significant difference nor correlation were reported between ear cleaning interval of domestic cat and infection with A.niger .

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reported between Ear cleaning interval of domestic cat and infection with A.niger.

Bathing frequency as a possible Risk Factor

As shown in table (4), cat with bathing frequency of more than one time per month represent 85/106, (80.18%) compared with 21/106, (19.81%) for those with bathing frequency of less than one time per month. A. niger was isolated primarily from those with bathing frequency of more than one time per month, 20/106, (18.86%) compared with 2 /106, (1.88%) from ear of those with less than one time per month. No significant difference nor correlation were reported between bathing frequency of domestic cat and infection with A.niger .

Season as a possible Risk Factor

As shown in table (4), cat attended to veterinary clinic in autumn months represent 59/106 (55.66%) compared with 47/106, (44.33%) for those presented in winter. A. niger was isolated primarily from those attended to veterinary clinic in autumn months, 15/106, (14.15%) compared with 7 /106, (6.60%)from ear of those presented in winter months. No significant difference nor correlation were reported between Season and infection with A.niger .

Table (4): Body weight, Hair length, Ear cleaning interval, Bathing frequency and Season as A Possible Risk Factors Associated with *A. niger* Infection among domestic cats.

Parameters		Fungal growth			χ2	P value	R	P value
		no growth	A. niger	Total				
Body weight (Gram)	300 -1250	49(46.22%)	12(11.32%)	61(57.54%)	26.216	0.291	0.005	0.962
	1251-2051	21(19.81%)	6(5.66%)	27(25.47%)				
	2052-2852	0(0%)	1(0.94%)	1(0.94%)				
	2853-3653	8(7.54%)	1(0.94%)	9(8.49%)				
	3654-4454	2(1.88%)	2(1.88%)	4(3.77%)				
	4455- 5255	2(1.88%)	0(0%)	2(1.88%)				
	5256 -6056	2(1.88%)	0(0%)	2(1.88%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				
Hair length	short hair	7(6.60%)	4 (3.77%)	11(10.37%)	1.818	0.178	-0.131	0.181
	longhair	77(72.64%)	18(16.98%)	95(89.62%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				
Ear cleaning interval	More than 2 weeks	59(55.66%)	18(16.98%)	77(72.64%)	1.176	0.278	-0.105	0.282
	less than 2 weeks	25(23.58%)	4 (3.77%)	29(27.35%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				

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Bathing frequency	more than one time per month	65(61.32%)	20(18.86%)	85(80.18%)	2.008	0.156	0.138	0.159
	less than one time per month	19(17.92%)	2(1.88%)	21(19.81%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				
Season	autumn	44(41.50%)	15(14.15%)	59(55.66%)	1.764	0.184	0.129	0.188
	Winter	40(37.73%)	7(6.60%)	47(44.33%)				
	Total	84(79.24%)	22(20.75%)	106(100%)				

Detection of FUM1 gene of Fumonisin and PKS gene for Ochratoxin in A. *niger* isolated from Ear of Domestic cats.

As shown in Table (5) and Figure (4), the FUM1 gene of fumonisin was detected in 7/17(41.17%) of A. niger isolated from ear of domestic cats. PKS gene for Ochratoxin was not produced by any isolates of A. niger, 0/17, (0%) as shown in figure (5).

Table (5): Frequency of PCR based detection for FUM1 gene for fumonisin and PKS gene for Ochratoxin produced by A. niger Isolated from ear of domestic cat

Source of A. niger	External ear of Domestic cat
Frequency of detection of FUM1 for fumonisin using sequence specific primers	7/17(41.17%)
Frequency of detection of PKS for Ochratoxin using sequence specific primers	0/17, (0%)

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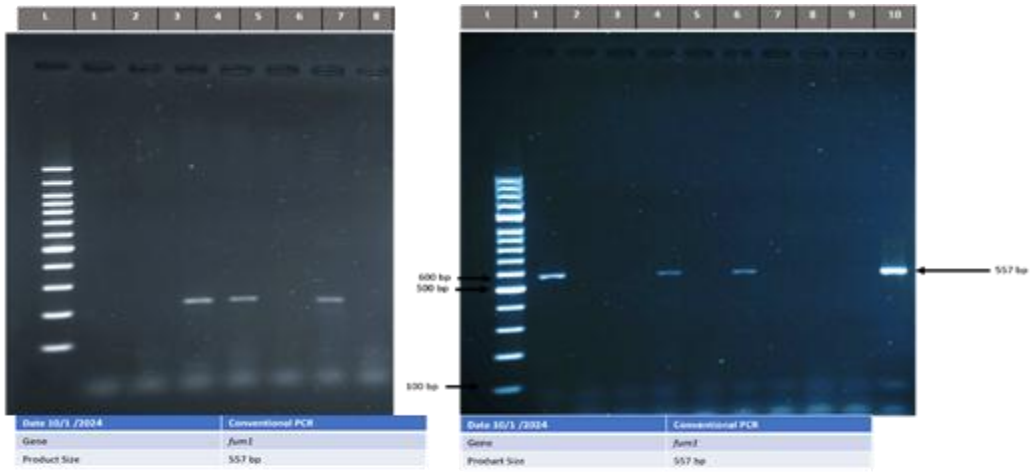

Figure (4): "DNA products of cat A.niger isolate fumonisin generated through fum1 gene primers, stained with Red safe dye. L : Ladder marker; lanes 4,5,7 (557bp) , fumonisin"

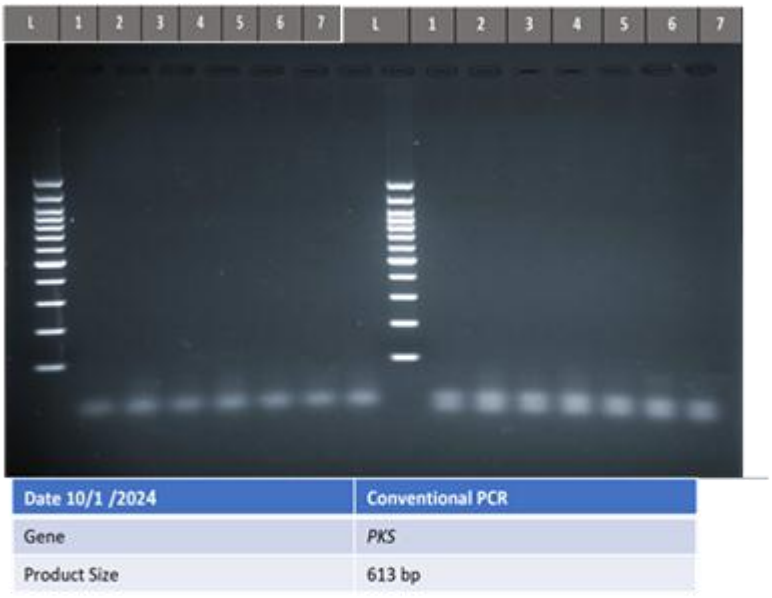

Figure (5): "DNA products of cat A.niger isolate Ochratoxin generated through pks gene primers, stained with Red safe dye. L : Ladder marker; lanes 1-10 ,DNA products"

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Discussion

Fungal Isolation from Ear swabs of domestic cats

The isolation of *A. niger* from the ears of domestic cats was done in two methods firstly morphological identification according to morphological features on SDA, and the results showed 22 *A. niger* isolated out of 106 total ear swab accounting (20.75%) of cultures samples. Then all *A. niger* isolated confirmed by conventional PCR using species-specific PCR primer pairs for *A. niger* (NIG1) and (Cmd A).

Two pairs of species-specific primers for the PCR identification of A. niger were developed by (Susca et al., 2007). The primers generated species-specific PCR products with lengths of 245 bp for A. niger. This feature is particularly advantageous for the potential development of a multiplex PCR assay. The sensitivity of this assay was determined to be approximately 10 pg of DNA in a 25 µl PCR reaction volume, using pure total DNA from both species. This method offers a rapid and reliable approach for detecting the presence of this ochratoxigenic species.

In accordance with current study (Mokhtar, 2022) showed that *A. niger* was isolated from 25% of cats. While the study of(Zenad *et al.*, 2015)showed that *A. niger* was isolated from (4.2%) of total cats skin samples which reflect the possibility of ease transmission of fungal spores to the external ear and germination takes place favored with humidity of local ear environment.

While Aspergillus species have been previously reported in cats otomycosis infections, A. fumigatus is generally considered the most common isolate(Goodale et al., 2016). However, current results suggest that *A. niger* may play a more significant role in cats otomycosis than previously recognized. This aligns with some earlier findings in canine otitis, where *A. niger* has been isolated, albeit less frequently than A. fumigatus (Goodale et al., 2016).

The prevalence of *A. niger* in current study was (20.75%) which was higher than what has been reported in most previous studies on feline aspergillosis. For instance, (Barrs V. R. & Talbot, 2014)reported *A. niger* as a causative agent in Sino-nasal aspergillosis in cats, but it was not among the most common isolates. The observed difference in As-

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pergillus species prevalence could stem from various factors. Geographical variations might play a role, as different regions may harbor distinct species. Additionally, current study concentrated on otomycosis, contrasting with prior research that predominantly examined sinonasal infections (Fawsitt et al., 2023).

Possible risk factors associated with *A. niger* infection among domestic cats

Age, sex, and weight of domestic cats

The present study investigated the age of domestic cats as a potential risk factor for *A. niger* infection in cats' ears. The results indicated no significant relationship between the age of the cats and the occurrence of *A. niger* infection. However, there was a non-significant slight tendency for infections to occur in the 2-6 months age group. These results are in concordance with the literature, where age is not considered a risk factor for aspergillosis in cats in most cases. Based on the ABCD guidelines on aspergillosis, the authors have not identified any predispositions to Aspergillus infections in cats regarding age or sex (Hartmann et al., 2013)

The slight positive correlation of *A. niger* infection with the age of the cats noted in this study where most of the affected cats were young (2-6 months) could be due to the fact that young animals have relatively immature immune systems that may make them susceptible to opportunistic infections (Burns-Naas *et al.*, 2008).Nevertheless, this was not statistically significant and hence the age alone cannot be used as a strong pointer to the chances of getting infected with *A. niger* in feline otomycosis. Likewise, the results of the analysis of the sex as the risk factor did not indicate any association between the infection with *A. niger* and the sex of the cats. A non-significant trend in the prevalence of infections in males as compared to females was noticed. This finding is in concordance with some prior studies conducted on aspergillosis in cats in which the predisposing factors for the disease have not been found to include sex (Barrs V. R. & Talbot, 2014). However, it must be mentioned that the majority of the previously published data regarding feline aspergillosis are devoted to systemic or sinonasal forms of the disease rather than otomycosis.

The present work aimed at examining the frequency of Aspergillus niger in the ears of domestic cats depending on

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their weight. Surprisingly, the highest isolation rate was recorded from the cats in the weight range of 300-1250 grams; however, no association was noted between the cats' body weight and *A. niger* infection.

The lack of a significant correlation between body weight and *A. niger* infection suggests that other factors may play a more crucial role in determining susceptibility to otomycosis in cats. This observation aligns with previous studies on fungal infections in animals, where factors such as immune status, environmental conditions, and concurrent diseases were found to be more influential than body weight. The higher isolation rate in cats weighing 300-1250 grams typically corresponds to young or small-breed cats, which may have different physiological or behavioral characteristics that could influence their susceptibility to *A. niger* colonization. For instance, younger cats might have less developed immune systems or different grooming habits that could affect the microbial ecology of their ears (Burns-Naas et al., 2008)Breeds of domestic cats

Fascinating trends are observed in the infection rates related to *A. niger* in the various breeds of cats, but the authors did not find a significant relationship between the breed and the rate of infection. Persian and Himalayan cats were the most frequently presented breeds during veterinary visits in current study, accounting for 31%. This high representation is in tune with the global prevalence of these breeds known as brachycephalic breeds(Barrs V. *et al.*, 2012; Hartmann et al., 2013). However, they were prevalent in the study population could also be an indication that these breeds are more prone to seek veterinary services from veterinarians due to some specific breed associated diseases (O'Neill *et al.*, 2019).

In the current study, Persian cats were the most represented breed in the overall ear samples; however, the highest percentage of *A. niger* isolation was identified in Himalayan cats (8. 49%), while Persian cats scored 6. 60%. The fact that the prevalence of A.niger otomycosis was higher in Himalayan cats compared with other breeds, even though the latter is slightly more numerous in the study sample, indicates that other factors may be contributing to A. niger infection rather than just the effect of breed . The higher prevalence of *A. niger* in brachycephalic

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breeds such as Himalayan and Persian cats is also in support with the previous findings that reported higher incidence of feline upper respiratory tract aspergillosis in these breeds (Cormack *et al.*, 2021).In current study, A.niger otomycosis in Shirazi breed revealed the third highest incidence , (3. 77%). Out of all the breeds, Calico and Sphynx breeds had the least number of *A. niger* isolation with a percentage of 0. 94% each. In the case of Sphynx cats, this low incidence is especially noteworthy given the fact that the Sphynx breed is characterized by a hairless phenotype which could conceivably change the conditions in the ear canal (Gandolfi *et al.*, 2010).

Hair length of domestic cats

The present study investigated the effect of hair length in domestic cats as a risk factor in occurrence of *A. niger* infections. The findings showed that cats with long hair were the most prevalent, representing 89 percent of all the cats. Thus, 62% (95 out of 106) of the sample, in contrast to short-haired cats that accounted for 10%. 37% (11 out of 106). *A. niger* was isolated mainly from the ears of long-haired cats (16. 98%) while it was isolated from only 3. However, it was observed that there was no difference or relationship between hair length and infection by A. niger. These findings are in agreement with other research showing that hair length is a factor in the occurrence of some types of fungi, but it is not necessarily a predictor of A. niger. It is also important to note that long-haired cats are more vulnerable to dermatophytosis and other fungal infections because the fur creates a dense and often moist environment, which is ideal for the development of fungal spores (Sattasathuchana *et al.*, 2020; Niae *et al.*, 2021) .

Frequency of ear cleaning and bathing of domestic cats

The current study focused on the level of cleaning of the ears of the cats as well as the possible correlation with *A. niger* infection. It was found that most of the cats (72. 64%) were cleaned more often than two weeks while the rest about (27. 35%) were cleaned less often than two weeks.

A. niger was isolated more often from cats with the interval between ear cleaning more than two weeks (16. 98%) than those with the interval of less than two weeks (3.77%). However, as earlier noted, there was no significant difference in the

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intervals of time the ears were cleaned and the occurrence/ prevalence of *A. niger* infection.

The findings of the present work suggest that cats, which are bathed more often, have a greater incidence of A. niger. In detail, the cats that were bathed more than once in a month only constituted 80 percent. Thus, 18 percent of the sample population, compared to 19 percent of the overall population, reported that their health had worsened. Among the respondents 81% for those bathed less frequently. Most importantly, *A. niger* was isolated from 18. The majority of cats were bathed more than once a month, 86% of them, while 1% of the cats were bathed only once a month. Ninety percent of the cats bathed less than once a week tested positive for the fungus.

The findings of the present study imply that there is a possibility of a correlation between the use of bathing as a cleaning practice and the occurrence of *A. niger* in the ears of domestic cats. As a result of constant washing, the ears' natural flora and moisture content are upset, providing a favorable environment for the fungus. This is in agreement with the practices of human medicine in which moisture and the alteration of the normal flora are considered to be causes of otomycosis (Hartmann et al., 2013; Mokhtar, 2022). But, as the above-presented trend shows, the further analysis did not show the difference or the correlation between the frequency of bathing and the infection rates of A. niger.

Season of infection

Regarding the seasonality of *A. niger* isolated from cat ears, the current study revealed that it was more frequent in autumn than in winter. Specifically, 14. Autumn was the most frequent season of presentation of cats with *A. niger* otomycosis at 15% followed by summer at 6%. About 60% were impacted in winter. However, the result of the analysis of variance indicated that there was no significant difference at the probability of 0. 05 or less, or correlation between the season and infection with A. niger.

The conditions favorable to the growth of fungi are also likely to be available in autumn such as moderate temperatures, high humidity. These factors may lead to increased presence of the spores in the environment hence increasing chances of getting infected (Bojanović *et al.*, 2023).

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Detection of Ochratoxin and Fumonisin for *A. niger* isolated from ear of domestic cat

The results of this study indicate that a significant proportion (41.17%) of Aspergillus niger isolates from the ears of domestic cats are capable of producing the mycotoxin fumonisin. This is concerning as Fumonisins are potentially carcinogenic and can cause various health issues in both animals and humans (Frisvad *et al.*, 2011). The finding that none of the *A. niger* isolates produced ochratoxin A is somewhat reassuring, as ochratoxins are also potent mycotoxins (Frisvad et al., 2011)

Previous studies have reported the isolation of Aspergillus species, particularly A. fumigatus, from the upper respiratory tract and ears of cats (*Goodale et al*., 2016). However, to the best of our knowledge, this is the first study to specifically investigate the mycotoxin production potential of *A. niger* isolates from feline otomycosis cases. The high prevalence of fumonisin-producing *A. niger* strains in this study is consistent with reports that *A. niger* has the potential to produce Fumonisins and ochratoxins, two groups of potentially carcinogenic mycotoxins (Frisvad et al., 2011)

Conclusions

A. niger associated otomycosis represent serious problem in cat, although otomycosis do not correlated with age, sex; body weight; breed; hair length; ear cleaning interval; bathing frequency of domestic cat and the season. Females appear to be at risk of getting A. niger infection. fumonisin was prominent fungal toxin produced by A. niger as FUM1 gene was primarily detected and associated with otomycosis.

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