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Correlation between *Aspergillus fumigatus* Isolated From Mouth , Nose and Ear of Hunting Dogs and Unusual Clinical Manifestations

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Abstract

Aim: Isolation of *A. fumigatus* from hunting dogs In Diyala province –Iraq , evaluation of relationship between *A. fumigatus* infection and Clinical Manifestations among hunting dogs .

Methods: Ninety nine swabs from mouth, nose and ear of hunting dogs with respiratory signs in Diyala province were included. Samples were cultured on Sabouraud Dextrose Agar. *A. fumigatus* was identified according to morphological features and molecular analysis **.**

Results: *A.fumigatus* was isolate from mouth ,nose and ear of hunting dogs, respectively . Unilateral ear drooping ,head tilting negatively associated with *A.fumigatus* isolation from mouth . Pruritis, Rub against wall, Erythematous ceruminous, Erythematous lesion, Malodorous, Ulcers around the nostrils, Lack of pigment or tissue and Lethargy were negatively correlated with *A.fumigatus* isolation from nose.

Conclusions : *A. fumigatus* infection represent serious problem for hunting dogs . Mouth, nose and ear respectively exposed to *A. fumigatus* with certain clinical manifestation .

Keywords : Molecular identification; *A. fumigatus* Clinical manifestations, hunting dogs

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Introduction

In recent years, aspergillosis opportunistic infections have been recognized as an important cause of morbidity and mortality in developing as well as developed nations [1, 2]. Aspergillosis is reported with increasing frequency in humans and animals from many regions of the world[3]. There are about 600 species of Aspergillus, of which about 27 species of Aspergillus are found to be associated in various clinical disorders of humans and animals (Pal., et al. 2014).

Disease is primarily caused by A.fumigatus, opportunistic filament forming moulds ;however, other species such as *A. amstelodami, A. candidus, A.chevallieri, A.clvatus, A.deflectus, A.flavus, A.glaucus, A.nidulans, A.niger, A.ochraceous, A.restrictus, A.syowii, A.tamari, A.terreus, A.udagawae, A ustusand A. versicolor* are also incriminated in the etiology of disease [3, 4] These fungi are widely prevalent in environment and are recovered from soil, air, water, plant substrates [5] . *A. fumigatus* secretes extracellular enzymes, most of them proteases, that degrade and recycle organic matter in the environment, but during infection they could serve to break down the structural barriers of the host and to obtain nutrients[6]. One of the host antimicrobial mechanisms is nutrient deprivation, and the amount of secreted hydrolases encoded on the genome may allow *A. fumigatus* to obtain nutrients from mammalian tissues without the need to activate the autophagic network. Some of these proteases can degrade collagen and elastin, which are the main components of the lung matrix[7] . *A. fumigatus* also secretes phospholipases, which break the ester bond of phosphoglycerides and thus may destabilize the host cell membranes causing cell lysis[8] .

The current study aims to isolation of *A. fumigatus* from hunting dogs In Diyala province –Iraq , identification of *A. fumigatus* by phenotypic characterization and PCR technique , evaluation of relationship between *A. fumigatus* infection and possible risk factors among hunting dogs

Materials and Methods

Study Area And Study Population

This study was performed in Baqubah city -Diyala province 33°45'34.71"N; 44°36'23.97"E ,Northeast . The study included 33hunting dogs , age ranged (0.6-7.1years) ,from 14 of October 2018 to 13 of January 2019. This study was conducted according to the principles of Helsinki declaration. A full explanation about the purpose of this study to all patients was done. Dully filled consent form obtained from all hunting dogs owners that agree to participate in the study. Approval of ethical review committee of college of veterinary medicine, university of Diyala ,Iraq was taken before initiation of the work .

Sample Collections :

Ninety nine Samples used were mouth ,nose and ear swabs . Sterile, clean swabs were used for sample collection, swabs was covered, to prevent contamination of air pollution and retention of the sample without contamination until cultured within a few hours if necessary[9, 10]. Cotton swabs were wetted with 0.85% normal saline then used to wipe the mouth, nose and ear several times, and then the swabs conducted for direct smear and culture purposes .

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Culture:

Swabs were inoculated into Sabouraud's dextrose agar(SDA), containing 0.05gm\L chloramphenicol, Penicillin at a concentration of 0.4 ml\L and Streptomycin at a concentration of 2 ml\L . The media were incubated at 37°C for 1-2 weeks[11, 12]

Staining

Lacto phenol cotton blue staining solution is added on a slide. By sterilized needle, a mycellial mat was transferred on fluid and pressed gently, then mixed with the stain. A clean cover slip had been taken and with the help of a forceps places the cover slip on mycellial mat. Observed under low to high power objectives of microscope [9, 13]

Molecular Identification of Aspergillus spp. by polymerase chain reaction

DNA Extraction:

DNA was extracted from *Aspergillus spp.* By using Wizard® genomic DNA Purification kit (Promega, USA) according to the protocol stated by the kit manufacturer **[14]**

Concentration and purity of DNA :

DNA was extracted from hundred isolated of *Aspergillus* spp. and they were concentrated in one tube. The concentration and the purity of the DNA samples were determined by Quantus Fluorometer at (9.9 ng/µl and 57 ng/µl) was used to detect the concentration of extracted DNA in order to detect the goodness of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted Quanty Flour Dye was mixed. After 5min incubation at room temperature, DNA concentration values were detected, according to the protocol stated by the kit manufacturer [14, 15]

Primer selection and preparation

Universal primers ITS1 (5`- TCCG-TAGGTGAACC TGCGG-3`) and ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) were used for detection of Aspergillus (Promega, USA) .

PCR working solution :

Optimization of PCR was accomplished after several trials. Thus the following mixture was adopted amplification reactions were produced in the 25µl final volume containing 12.5 µl Go Taq® master mix (Promega, USA), 2µl of the primers and 2µl DNA template and complete the volume by 8.2 ul nuclease-free water

Programmable thermal controller

Program for amplifying the 5.8S rDNA and the ITS 1region, amplified from type of ITS1 and ITS3 for *Aspergillus* spp. For identification of *Aspergillus* spp. ,an initial denaturation step at 95°C for five minutes was followed by thirty cycles of denaturation at 95°C for thirty seconds, annealing at 55°C for thirty seconds , and extension at 72°C for thirty seconds, with a final extension step of 72°C for seven minutes **[16].**

Agarose Gel Electrophoresis:

After PCR amplification, agarose gel electrophoresis was adopted to confirm the presence of amplification. PCR was completely dependable on the extracted DNA criteria, according to the protocol stated by the kit manufacturer (Promega, U.S.A)

Statistical Data Analysis: Patients demography and cross tabulation were calculated by Statistical Package for the Social Sciences for Windows version 17 (SPSS, Armonk, NY: IBM Corp.). Pearson's chi-square and Pearson's correlation coefficient were used for correlation between the variables of the two tests. P value of ≤ 0.05 and ≤ 0.01 (two tailed) was set to be statistically significant [21-23]

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Results

Table (1) shows the identification of *A.fumigatus* isolated from hunting dogs according to morphological features on SDA .The total number of *A.fumigatus* isolated from hunting Dogs was (17.17%) . *A.fumigatus* was isolated from 10/33 (30.30%) mouth swabs ,(12.12%) nasal swabs and (9.09%) ear swabs. All 17 *A.fumigatus* positive samples , (17.17%) confirmed by conventional PCR and sequencing. Analysis of sequences and confirmation of *A.fumigatus* homogenic data using rRNA database (NCBI) after amplification of fungi's ribosomal RNA. All processes including fungi gDNA extraction, PCR amplification, sequencing, and assembly. For fungi, analysis on ITS region (18S); length greater than 500 bp guaranteed to be *A.fumigatus* as shown in figure (1).

Possible Correlation Between Clinical Signs And Isolation Of .fumigatus From Mouth of Hunting dogs

Table (2) revealed no significant correlation was reported between Pruritis ; head shaking ; rub against wall ; blackish waxy discharge ; erythematous ceruminous ; erythematous lesion ; malodorous; greenish yellow nasal discharge; ulcers around the nostrils; pain around nose ; Sneezing ; lack of pigment or tissue ; bleeding around lesion ; lethargy and isolation of *A.fumigatus*

Possible Correlation Between Clinical Signs And Isolation Of *A.fumigatus* From Nose of Hunting dogs

Table (3) revealed that significant correlation was reported between Pruritis ; rub against wall ; erythematous ceruminous ; erythematous lesion ; malodorous ; ulcers around the nostrils; lack of pigment or tissue lethargy and isolation of *A.fumigatus* from nose of hunting dogs (p value =.004; 0.012; 0.001; 0.007; 0.012; 0.039; 0.022; 0.022) respectively.

No significant correlation was reported between head shaking; head tilting ; blackish waxy discharge ; unilateral ear drooping ; greenish yellow nasal discharge ; Sneezing ; bleeding around lesion and isolation of *A.fumigatus* from nose of hunting dogs (p value= 0.083; 0.083; 0.092; 0.092; 0.212; 0.515; 0.128) respectively.

Possible Correlation Between Clinical Signs And Isolation Of A.fumigatus From Ear of Hunting dogs

Table (4) revealed No significant correlation was reported between Pruritis ; head shaking ; head tilting ; rub against wall ; blackish waxy discharge ; erythematous ceruminous; erythematous lesion ; malodorous ; unilateral ear drooping; greenish yellow nasal discharge ; ulcers around the nostrils ; pain around nose ; Sneezing ; lack of pigment or tissue ; lethargy and isolation of *A.fumigatus* from ear of hunting dogs (p value =.604; 0.408; 0.491; 0.389; 0.266; 0.491; 0.326; 0.251; 0.912; 0.711; 0.373; 0. 319; 0.580; 0.812; 0.115) respectively . Significant correlation was reported between bleeding around lesion and isolation of *A.fumigatus* from ear of hunting dogs (p value= 0.024).

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Table (1) :identification of *A.fumigatus* isolated from hunting dogs according to morphological features on SDA

sample for hunting Dogs	Isolation status on SDA		Total No. of swabs
	No growth	***A.fumigatus***	
Mouth	**23(69.7%)**	**10(30.3%)**	**33(100%)**
Nose	**29(87.9%)**	**4(12 .12%)**	**33(100%)**
Ear	**30(90.9%)**	**3(9.09%)**	**33(100%)**
Total	**82(82.83%)**	**17(17.17%)**	**99(100%)**

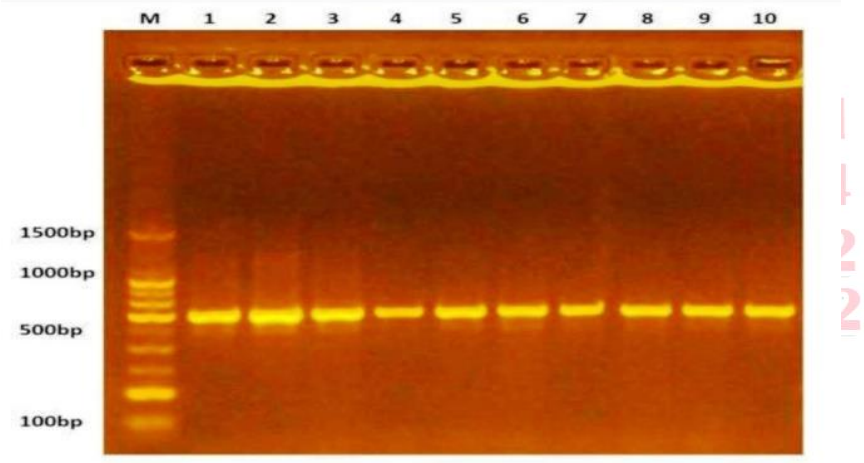

Figure (1):DNA products of *A. fumigatus* generated through ITS1 (5`- TCCG-TAGGTGAACCTGCGG-3), and ITS2 (5-TCCTCCGCTTATTGATATGC-3) primers, stained with Ethidium bromide. M : Molecular marker (100bp); lanes 1-10 (517bp) , *A. fumigatus* .

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Table (2): Possible Correlation Between Clinical Signs And Isolation Of *A.fumigatus* From Mouth of Hunting dogs

Clinical Signs		*A.fumigatus* infection No.(%) of hunting Dogs Mouth	R	P Value
Pruritis	positive	3(9.09%)	-.142	.431
	Negative	7(21.21%)		
Head shaking	positive	2(6.06%)	-.031	.864
	Negative	8(24.24%)		
Head Tilting	positive	4(12.12%)	-.373	.033
	Negative	6(18.18%)		
Rub against wall	positive	5(15.15%)	-.101	.576
	Negative	5(15.15%)		
Blackish waxy discharge	positive	4(12.12%)	-.050	.783
	Negative	6(18.18%)		
Erythematous ceruminous	positive	2(6.06%)	-.031	.864
	Negative	8 (24. 24%)		
Erythematous lesion	positive	4(12.12%)	-.008	.964
	Negative	6(18.18%)		
Malodorous	positive	2(6.06%)	-.159	.376
	Negative	8(24. 24%)		
Unilateral ear drooping	positive	7(21.21%)	-.461	.007
	Negative	3(9.09%)		
Greenish yellow nasal discharge	positive	3(9.09%)	-.089	.624
	Negative	7(21.21%)		
Ulcers around the nostrils	positive	2(6.06%)	-.089	.622
	Negative	8(24.24%)		
Pain around nose	positive	4(12.12%)	-.242	.174
	Negative	6(18.18%)		
Sneezing	positive	1(3.03%)	-.021	.908
	Negative	9 (27. 27%)		
Lack of pigment or tissue	positive	3(9.09%)	-.040	.823
	Negative	7(21.21%)		
Bleeding around lesion	positive	4(12.12%)	-.008	.964
	Negative	6(18.18%)		
Lethargy	Positive	3(9.09%)	-.040	.823
	Negative	7(21.21%)		

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Table (3): Possible Correlation Between Clinical Signs And Isolation Of *A.fumigatus* From Nose of Hunting dogs

Clinical Signs		*A.fumigatus* infection of hunting Dogs Nose No.(%)	R	P Value
Pruritis	positive	3(9.09%)	-.489	.004
	Negative	1(3.03%)		
Head shaking	positive	2(6.06%)	-.306	.083
	Negative	2(6.06%)		
Head Tilting	positive	2(6.06%)	-.306	.083
	Negative	2(6.06%)		
Rub against wall	positive	4(12.12%)	-.433	.012
	Negative	0(0%)		
Blackish waxy discharge	positive	3(9.09%)	-.298	.092
	Negative	1(3.03%)		
Erythematous ceruminous	positive	3(9.09%)	-.547	.001
	Negative	1(3.03%)		
Erythematous lesion	positive	4(12.12%)	-.461	.007
	Negative	0(0%)		
Malodorous	positive	2(6.06%)	-.431	.012
	Negative	2(6.06%)		
Unilateral ear drooping	positive	3(9.09%)	-.298	.092
	Negative	1(3.03%)		
Greenish yellow nasal discharge	positive	2(6.06%)	-.223	.212
	Negative	2(6.06%)		
Ulcers around the nostrils	positive	2(6.06%)	-.361	.039
	Negative	2(6.06%)		
Pain around nose	positive	1(3.03%)	-.007	.971
	Negative	3 (9.09%)		
Sneezing	positive	0(0%)	.117	.515
	Negative	4 (12.12%)		
Lack of pigment or tissue	positive	3(9.09%)	-.398	.022
	Negative	1(3.03%)		
Bleeding around lesion	positive	3(9.09%)	-.271	.128
	Negative	1(3.03%)		
Lethargy	Positive	3(9.09%)	-.398	.022
	Negative	1(3.03%)		

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Table (4): Possible Correlation Between Clinical Signs And Isolation Of *A.fumigatus* From Ear of Hunting dogs

Clinical Signs		*A.fumigatus* infection of hunting Dogs Ear No.(%)	R	P Value
Pruritis	positive	1(3.03%)	-.094	.604
	Negative	2(6.06%)		
Head shaking	positive	0(0%)	.149	0.408
	Negative	3 (9.09%)		
Head Tilting	positive	1(3.03%)	-.124	0.491
	Negative	2(6.06%)		
Rub against wall	positive	2(6.06%)	-.155	.389
	Negative	1(3.03%)		
Blackish waxy discharge	positive	2(6.06%)	-.199	.266
	Negative	1(3.03%)		
Erythematous ceruminous	positive	1(3.03%)	-.124	.491
	Negative	2(6.06%)		
Erythematous lesion	positive	2(6.06%)	-.177	.326
	Negative	1(3.03%)		
Malodorous	positive	1(3.03%)	-.206	.251
	Negative	2(6.06%)		
Unilateral ear drooping	positive	1(3.03%)	.020	0.912
	Negative	2(6.06%)		
Greenish yellow nasal discharge	positive	1(3.03%)	-.067	.711
	Negative	2(6.06%)		
Ulcers around the nostrils	positive	1(3.03%)	-.160	.373
	Negative	2(6.06%)		
Pain around nose	positive	0(0%)	.179	.319
	Negative	3 (9.09%)		
Sneezing	positive	0(0%)	.100	0.580
	Negative	3 (9.09%)		
Lack of pigment or tissue	positive	1(3.03%)	-.043	0.812
	Negative	2(6.06%)		
Bleeding around lesion	positive	3 (9.09%)	-.392	.024
	Negative	0(0%)		
Lethargy	Positive	2(6.06%)	-.280	0.115
	Negative	1(3.03%)		

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Discussion:

Current study proved that *A.fumigatus* was isolated from (17.17%) of hunting dogs according to according to morphological features on SDA as well as via conventional PCR using ITS1 and ITS4 . Current study was interested in the molecular identification of the isolated and morphologically characterized *A. fumigatus* from mouth, nose and ear of hunters and hunting dogs. To achieve this goal the present study utilized a common section of the fungal genome that includes the 18S, 5.8S, and 26S genes, which code for rRNA and whose nucleotide sequence is also relatively conserved among fungi. This section also includes the intervening ITS regions, called ITSI and ITS4, whose DNA sequences vary. Although the ITS-coding regions are not translated into proteins, they have a critical role in the development of functional rRNA; and because of the sequence variations of these regions among species, these regions show promise for use as signatures for molecular biology-based assays and identification of fungi under genetic level[17, 18] .

Current study come in line with others [19, 20]that using of ITS regions for amplification with The PCR technique *with common* section 18S, 5.8S, and 26S genes, coding for rRNA , was sensitive for the identification of Aspergillus. They stated the amplification product of the universal fungal primers, ITS1 and ITS4 was detected, therefore the larger amplicons served to confirm the presence of a fungal target[21] .

On the other hand current study agree with that reported by [22] that The ITS 1-5.8S- ITS 4 region was chosen for the design of genus and species-specific primers for identification of Aspergillus, and as a result of high nucleotide variability among genera and species, The ITS region is a good molecular target for species level identification [22]and is extensively used as a universal DNA barcode in fungal taxonomy studies [20].

A.fumigatus was isolated from (30.30%) mouth swabs ;(12.12%) nasal swabs (9.09%)from ear swabs .These result considered very low compared with that reported by [23], they stated that *A.fumigatus* was recovered by classical culture technique as well as via conventional PCR using ITS1 and ITS2 from (96.7%) of dogs with respiratory signs of sino-nasal infections. Also [23-26]stated that *A. fumigatus* is the most common etiological agent of canine sino-nasal aspergillosis". Current result come in accordance with that reported by [27] stated that *A. fumigatus* was isolated from 6.66% of otomycosis in dogs of Sulaimania province, Iraq. Current isolation rate was very low compared with that reported by [26, 28],stated that *A. fumigatus* causing sino nasal aspergillosis was recovered from 7-34 % of dogs with nasal disorders and is the second most common cause of chronic nasal discharge.

Current study revealed that *A.fumigatus* was the main fungus that isolated from hunting dogs that suffered from a number of clinical signs with significant correlation between these signs and fungal isolation .This result come in agreement with that reported by [25, 26, 29] ,they stated that "Sino-nasal aspergillosis (SNA) afflicts more frequently dolichocephalic dogs like the master of hunting dogs in Iraq (Saluki) as well as mesocephalic dogs ,where they

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commonly infected with *A. fumigatus* as a primary pathogen" which could be due to smaller sino-nasal surface area[30, 31]. Similar clinical notification that was in accordance with current study was reported by [23] . Exposure of dogs to *A.fumigatus* conidial spores which is distributed in soil ,water, air as well as decaying vegetation, and dust were quite common where the mucociliary apparatus and mucosal innate immunity of the upper respiratory tract were effective to prevent initiation of infection ,otherwise in case of heavy exposure to a small size *A.fumigatus* conidia as well as production of different mycotoxins including gliotoxin that prevent the activity of mucociliary system and facilitate respiratory colonization of *A.fumigatus* and subsequently the clinical presentation of sinusitis was inevitable[30, 31].

Current study proved significant correlation between some clinical signs and behaviors that appear on the dogs and the isolation of *A.fumigatus* from the mouth of hunting dogs. One of the most common clinical signs was unilateral ear drooping that was reported in (21.21%) of hunting dogs with positive isolation of *A.fumigatus* from their mouth followed by head tilting that was reported in (12.12 %) of hunting dogs . These presentations come in agreement with [27] ,they stated that dogs infected with *A.fumigatus* were presented with unilateral or bilateral dropping of ears .

Current study proved significant correlation between some clinical signs that appear on the dogs as well as behaviors and the isolation of *A.fumigatus* from the nose of hunting dogs. The common clinical signs were the presence of itching or pruritis in (9.09%) of hunting dogs , rubbing against wall in (12.12%); erythematous ceruminous (9.09%) ;erythematous lesion (12.12%) , malodorous (6.06%), ulcers around the nostrils (6.06%); lack of pigment or tissue , (9.09%) ; lethargy (9.09%) which come in agreement with that reported by [32],they stated that clinical signs are non-specific and include lethargy, weight loss, central nervous system signs, and ataxia due to musculoskeletal lesions. On the other hand the clinical observation of [25, 26, 33],come in line with current study ,in that the fungal infection in the nose of dogs usually caused by *A fumigatus* with main clinical signs such as lethargy, nasal pain, ulceration of the nares, sneezing, unilateral or bilateral sanguinopurulent nasal discharge, frontal sinus osteomyelitis, and epistaxis. Furthermore [26], stated that nasolacrimal duct may be destructed which leads to ocular discharge also neurologic signs occur if cribriform plate is affected .

Current finding come in accordance with that reported by [34],they stated that chronic nasal discharge from one nostril with a strong odor , sometime nose bleeds may occur intermittently with obvious inflammation and roughness and ulceration of the edges of the nostrils associated with breaking of nasal tissue and bleeding from breaks as well as pawing at the nose or face .On the other hand [35, 36] ,stated that "initial clinical signs are non-specific and include mucopurulent nasal discharges that may become hemorrhagic with eventual depigmentation of the nasal plane . Other study by [30]revealed that " nasal signs in case of sinonasal aspergillosis may be present for weeks to months or even years with chronic mucopurulent to purulent nasal discharge, nasal pain and nasal planum ulcera-

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tion, nasal planum depigmentation most commonly reported .while Sneezing, epistaxis, decreased appetite, signs of depression and seizures may be identified .Current study come in agreement with that reported by [27], they stated that dogs with otomycosis and positive *A.fumigatus* growth for swabs taken from them usually presented with unilateral or bilateral dropping of ears, head shaking, pruritus, pain when palpated, erythema and swelling of ear skin or of the ear canal with increased amount of cerumen" but unfortunately from statistical point of view the was no significant correlation between these clinical manifestations and *A.fumigatus* otomycosis except for bleeding around the lesion .This may attributed to the nonspecific nature of these signs that could be noticed in any injuries for dogs ear via foreign materials or infection.

Conclusion :

A.fumigatus was isolate from mouth ,nose and ear of hunting dogs, respectively . old age, males infected frequently with *A.fumigatus* . Unilateral ear drooping ,head tilting negatively associated with *A.fumigatus* isolation from mouth . Pruritis, Rub against wall, Erythematous cerumenous, Erythematous lesion, Malodorous, Ulcers around the nostrils, Lack of pigment or tissue and Lethargy were negatively correlated with *A.fumigatus* isolation from nose.

A. fumigatus infection represent serious problem for hunting dogs . Mouth, nose and ear respectively exposed to *A. fumigatus* with certain clinical manifestation . old age and males more vulnerable for infection with *A.fumigatus* among hunting dogs.

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