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Molecular Diagnosis of *Cladosporium sp.* and *Fusarium equiseti* Fungal Infection for Zoonotic Otitis externa from Formalin-fixed Samples

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Abstract:

A total of thirty otitis externa samples from the pet Shirazi cats and nineteen skin swab samples were collected from children who had direct hand contact with them between October 2024 and March 2025. Macroscopic Potato dextrose agar (PDA) cultures and microscopic characteristics were used to identify filamentous fungal species, and DNA was extracted from seven formalin-fixed fungal cultures using commercial purification kits for PCR-based phylogenetic analysis. Cultures initially developed fluffy white colonies, which turned brown to dark brown by the seventh day. Microscopic analysis displayed the presence of filamentous mycelia, hyphae, and conidia. Fungal species were isolated from 26.31 % (5 out of 19) of the children's samples and 33.3% (10 out of 30) of the pet cat samples. PCR enabled the identification of several fungal species, including *Cladosporium cladosporioides*, *Cladosporium limoniforme*, *Fusarium equiseti*, *Cladosporium cucumerinum*, *Cladosporium tenellum*, and *Stemphylium paludiscirpi*. All sequences matched identically with those listed in the NCBI GenBank database. In conclusion, this study demonstrates that *Cladosporium* and *Fusarium species* isolated from pet cats may pose a zoonotic risk to children, underscoring the importance of pet hygiene and public health awareness. *Cladosporium species* and *Fusarium equiseti* were found in six samples of otitis externa in cats kept as pets. Given that most children have close experiences with cats in their homes, which are typically infected with diseases in their pet cats, the results of this study have highlighted the importance of considering pet cats as an infectious source of this disease in 26.31% of patients.

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INTRODUCTION

Cladosporium has been found to be an etiologic agent in humans and other vertebrate hosts (Kaneda *et al*., 2023; Sandoval-Denis *et al*., 2015). Molecular research continues to shed light on its pathogenic potential, despite the fact that it is primarily recognized as a common endophyte or saprobic fungus prevalent in the environment. Sandoval-Denis et al. (2016) identified 10 novel species generated from clinical specimens of humans and animals from the United States based on molecular phylogenetic research and traits based on DNA sequences. The massive Ascomycota genus Cladosporium (Cladosporiaceae, Capnodiales) has more than 189 species, most of which are saprobes with a global distribution that have been isolated from a wide range of substrates (Bensch *et al*., 2012; Bensch *et al*., 2015; Crous *et al*., 2014; David, 1997). Our knowledge of the clinical and ecological significance of this complex genus is further enhanced by recent taxonomic revisions that continue to clarify the species boundaries within it (Sandoval-Denis *et al*., 2016; Woudenberg *et al*., 2017).

Several illnesses, including mycotic dermatitis, have been linked to Cladosporium spp. This genus, which belongs to the phylum Ascomycota, has species that can infect animals like marine tropical fish and cause opportunistic and systemic diseases in humans (Harms *et al*., 2006; Sandoval-Denis *et al*., 2016). One of the most commonly isolated environmental fungi in the world is Cladosporium. The conidia of these species are darkly colored, and their colonies might be green, brown, or black, growing in simple or branching patterns. The darkly pigmented mold species *Cladosporium cladosporioides* can be found all over the world on a wide range of indoor and outdoor substrates (Portnoy *et al*., 2004; Zeng *et al*., 2006). Although its function in the breakdown of organic matter is widely recognized, it has the potential to have a substantial negative influence on human health, especially as an allergen and in people with respiratory disorders or weakened immune systems (Kaneda *et al*., 2023). Although some reports claim that some species are only colonists, their extensive range and ongoing isolation from clinical specimens attest to their function as agents of opportunistic phaeohyphomycosis, which includes deep and subcutaneous infections in both humans and animals (Sandoval-Denis *et al*., 2015; de Hoog *et al*., 2011).

According to a Basra study, otitis externa affects 21.3% of cats, with *Pseudomonas aeruginosa* being a frequent infection. Children are also affected, albeit at a lesser rate of 9.3%. According to a study conducted in Karbala, Iraq, 45.99% of household cats had otitis externa (Kheseli *et al*., 2021; Bader *et al*., 2024).

Fusarium spp. are one of the most common filamentous fungi globally, known for causing a wide range of diseases in both plants and animals, including humans (Al-Jobory *et al.,* 2017; Zaynab *et al.,* 2017; Al-Deen and Al-Jobory, 2018; Hashmi *et al.,* 2018; Ekwomadu and Mwanza, 2023; Ghufran *et al*., 2025). Localized, locally invasive, or disseminated infections are caused by *Fusarium species* in humans. The primary clinical manifestations include onychomycosis, skin deterioration, and eye infections such as endophthalmitis and keratitis. The latter may be post-traumatic, result from contaminated contact lenses or mold-containing ophthalmic solutions, or occur in individuals using topical corticosteroids or antibiotics who have underlying corneal illness (Fariñas *et al*., 2012). Patients with severe neutropenia, especially those with severe neutropenia brought on by medications or hematologic neoplasms, are the most vulnerable (Hof, 2020). Among the fungal plant pathogens is the genus *Stemphylium* (Woudenberg *et al*., 2017).

Historically, morphological traits, both macroscopic and microscopic, have been used to identify fungal species. Common identification techniques include examining the colonies' characteristics and differences as well as morphological features such as conidial size, texture, form, and structure (Portnoy *et al*., 2004; Sterling and Lewis, 1998). Differentiating between fungi with similar morphologies is very challenging. Because of these worries, it is essential to create new fungal identification

procedures that are quick, accurate, simple, and economical. Many of the issues with morphological identification have been addressed by molecular biology techniques. The highest sensitivities and specificities were demonstrated by the nested PCR approach, which used two DNA purification kits to extract DNA from paraffin-embedded and formalin-fixed tissues (FFPE) for the detection of *Sporothrix species* in cat samples. A straightforward restriction fragment length polymorphism/polymerase chain reaction test that can detect filamentous fungi of medical significance (Faria *et al*., 2022; Dean *et al*., 2005).

The purpose of this study was to identify fungus isolates by employing both conventional and molecular methods on particular primers using formalin-fixed (FF) fungal species pellets.

MATERIALS AND METHODS

Ethical approval

This study received ethical approval from the Scientific and Ethics Committee of the Department of Pathology and Poultry Diseases, College of Veterinary Medicine at Al-Qasim Green University, Ministry of Higher Education and Scientific Research, Iraq (Protocol No. 2937, dated 10/11/2024). For children's samples, no identifiable information was contained to ensure anonymity. Pet cats' samples were collected following international animal care standards, handled, and managed according to the necessary security and biosafety procedures.

Full compliance with relevant international ethical guidelines was maintained.

Study period and location

From October 2024 to March 2025, this study was carried out in Iraq's Hilla region (Babil city).

Sampling and sample collections

Recruitment: Thirty swab samples were taken from pet Shirazi cats exhibiting clinical indications of otitis externa discharge.

The children were watched when they were with their family, inside the house. We documented direct interaction with the animal daily, such as handling, petting, and sleeping next to it. Nineteen skin lesion swab samples from close contact mixed-sex children between the ages of 11 and 14 years during the study period (6 months) represent all cases meeting the inclusion criteria.

Contact: Close contact is defined as two hours or more of daily direct physical contact.

In order to ensure that there was no environmental contamination during processing, sterile swabs were processed alongside clinical samples as negative controls, according to the statement. There were no growth indicators in any of the negative controls.

Replication: State if sub-culturing was done to ensure purity or if multiple cultures were used.

Clinical findings of fungal infection in cats and patients

Clinically, the infected cats show signs of itching and shaking of the head with exudate inside the ear. On the other hand, although the children's health status was not systematically evaluated for this study, parents stated that none of the participants had any known immunodeficiencies or long-term respiratory disorders. Lesions were commonly observed on itchy lesions on the hands locally and in females (Figure 1, a and b).

Fig. 1. a: Pet Shirazi white cats with fungal infection. **b:** Fixed cutaneous with erythematous borders of mycosis in humans.

Mycological examination

Culture and microscopic examination

Following the typical manufacturer's recommendations, the collected samples from pet cats and children were cultivated on potato dextrose agar (PDA). It was utilized since potato dextrose agar (PDA; Oxoid, UK) is a popular non-selective medium for cultivating a range of fungi.

The incubation was carried out at 25°C (± 0.5°C), which is the ideal temperature for most environmental fungi for growth. All cultured plates were then incubated for 7 days (Echevarría and Iqbal, 2021).

Plates were examined daily for fungal development; samples were considered negative in week four if no bacterial growth was found. The isolated sporophyte cultures were first identified by looking at their colony morphologies (macroscopically) and microscopic features on PDA. The front and back sides of the culture were examined for growth texture, color, and pigmentation in order to make a macroscopic identification. Using adhesive tape (Jarjees and Issa, 2022), the isolates were detected under a microscope. Following the application of the fungal-structured adhesive piece of tape to the slide, it was observed through a 40x lens.

Statistical analysis

IBM SPSS Statistics software (Version 26) was used to conduct the statistical study. Frequencies and percentages were used to display categorical variables. Because of the small sample size (N=49) and the occurrence of predicted frequencies below 5 in over 20% of the contingency table cells, Fisher's Exact Test was used to assess the prevalence of fungal infections between the two groups (pet cats and children).

Additionally, an analysis was conducted on the distribution of particular fungal species isolates (n = 9 detected isolates) among the host groups. As an extension of Fisher's Exact Test, a Monte Carlo simulation for the exact test (with 10,000 repeats) was utilized to generate a stable and trustworthy p-value given the sparse data matrix containing numerous rare species.

PCR procedure

Pellets of seven out of ten positive samples were reference strains of previously known fungal species — *Cladosporium limoniforme*, *Cladosporium cladosporioides*, *Cladosporium cucumerinum*, *Fusarium equiseti*, *Cladosporium tenellum*, and *Stemphylium paludiscirpi*—were employed in the investigation. The pellets from the harvested mycological culture were centrifuged and then preserved in 10% buffered formalin (Carson and Cappellano, 2015).

Sequencing method

PCR amplicon nucleic acid sequencing

Commercial forward and reverse sequencing of the resolved PCR amplicons was performed in accordance with the instruction handbook from the sequencing company (Macrogen Inc., Geumchen, Seoul, South Korea). The Analysis was restricted to ABI clear chromatographs. (Applied Biosystem) files in sequence to make sure that the variations and annotation weren't the outcome of sequencing artifacts or PCR. The obtained PCR fragments' virtual positions and further details were ascertained by comparing the recovered nucleic acid sequences with the observed nucleic acid sequences of nearby samples.

The sequence analysis data

Utilizing Version 7.1 of the BioEdit Sequence Alignment Editor Software (DNASTAR, Madison, WI, USA), forward primer ITS (5' TCCGTAGGTGAACCTGGGG 3') and reverse primer ITS (5' TCCTCCGCTTATTGATATGC 3') were used in PCR reactions. The order of findings of the PCR items from the samples that were targeted was adjusted, matched, and examined to match the relevant sequences within the reference repository. This was carried out in compliance with the procedure outlined by Al-Shuhaib and Hashim (2023). The variations found in each sequenced sample were numbered in the corresponding place within the

reference genome as well as in the PCR amplicons. In the amplicons of the PCR and at the corresponding locations, the nucleic acids found in the reference genome were numbered. The strains used were: *Fusarium equiseti*, *Cladosporium cladosporioides*, and *Stemphylium paludiscirpi*. Sequences discovered in the samples were annotated using Version 4.0.4 of SnapGene Viewer (https://www.snapg ene.com).

Sequences being added to GenBank

According to Benson et al. (2016), all sequences that had been examined and evaluated were uploaded to the NCBI Bankit portal, and all directions provided by the site were adhered to. For the researched sequences to be assigned a unique GenBank entry number, the submitted sequence was submitted as nucleic acid sequences to the NCBI.

Building a complete phylogenetic tree

The neighbor-joining technique was used in this work to construct a specific comprehensive tree. The discovered variants were compared to their adjacent homologous reference sequences using the BLASTn NCBI server (Zhang *et al.,* 2000). Then, employing the neighbor-joining method, a complete inclusive tree was constructed, incorporating the noted variant, as well as the BioEdit program (v7.2.5), which displayed it as a rectangle.

RESULTS

Isolation and microscopy

Fungal growth in white colonies, fluffy and dense filaments at the top of the plate; on the reverse side, there are creamy white roots (Figure 2, a-d). Microscopically, without the need for any kind of dyes, fungal species sporangium structure, which revealed the presence of the filamentous mycelia, developed thin, hyaline, septate, branching hyphae with right-angled conidiophores growing at their globose apices to generate small, oval, globose, pyriform, hyaline, or dark conidia that are grouped in clusters that resemble a flow-rosette (Figure 3, a- d).

Fig. 2. The macroscopy culture. **a:** After 48 hrs. White- fluffy fungal species colonies. **b, c:** The spread culture in third- day from top and creamy roots in back. **d:** After seven days, the colonies become brown to dark green in color with their branches.

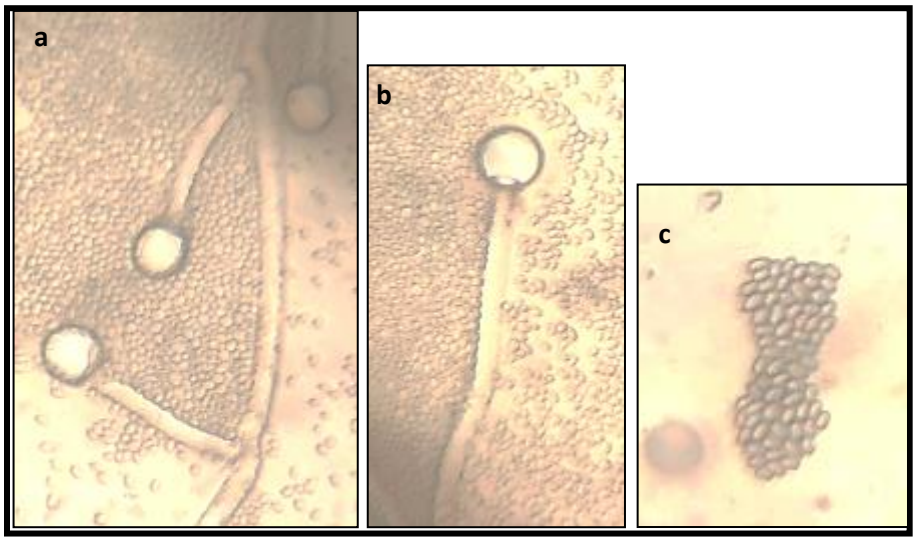

Fig. 3. The microscopy culture 40×. **a:** The branching hyphae, septate, hyaline filamentous mycelia. **b:** The mycelium is tube-like with a dark-halo hyaline conidiophore and rosette-oval conidia. **c:** The cluster-nest conidia.

In this study, the first colonies are white, smooth, and humid. They turn from brown to black over time, usually within seven days, due to the production of melanin characteristic of dematiaceous fungi.

33.33 percent (10/30) of the isolated fungal species on PDA plates were from pet cats, while 26.31 percent (5/19) were from children (Table 1).

Table 1. The rates of isolation of fungal species from pet cats' otitis externa and children's skin on PDA.

Samples source	**fungal *spp.***		
Host	No. of negative	No. of positive	%
Pet Cats p= 30	20	10	33.33
Children p= 19	14	5 (1 male, and 4 females)	26.32
Total p= 49	34	15	7.01
p-value=			0.749†

†Fisher's exact test p-values
PDA= Potato Dextrose agar

PCR detections

The PCR approach demonstrated 100% specificity for fungal species by detecting the 600-500 bp portion of all seven fungal species examined in FF samples of pellets using the DNA extraction process. Two of them (accession number: OR272195.1) were linked to *Fusarium equiseti*, one to *Stemphylium paludiscirpi* (accession number: MK460857.1), and four (accession numbers: PP556568.1, GQ458030.1, GU594746.1, and KP701932.1) to *Cladosporium species* in GenBank (Table 2) (see figures and tables for samples S1, S2, S3, S4, S5, S6, S7 Files for more details).

Table 2. The identified fungal species by PCR.

***Cladosporium cladosporioides* p= 7**	***Cladosporium limoniforme* p= 7**	***Cladosporium cucumerinum* p= 7**	***Cladosporium tenellum* p= 7**	***Fusarium equiseti* p= 7**	***Stemphylium paludiscirpi* p= 7**	**Total isolates**
1 (14.29%)	1 (14.29%)	1 (14.29%)	1 (14.29%)	2 (28.57%)	1 (14.29%)	7 (100%)
p-value=						0.995-1.000

Following the successful amplification of a 500 bp fragment from each isolate using a universal fungal rRNA primer pair, the PCR results generated from DNA recovered from isolated fungal species are displayed in Figure 4.

ML phylogenetic analysis showed that the examined Cladosporium species (S1, S2, S6, S7) and isolates of *F. equiseti* (S3, 4) clustered with Aspergillus sp., *Penicillium citrinum*, and other Cladosporium species. However, the sequences of the isolates of Fusarium and Cladosporium spp. and *F. equiseti* that were retrieved from the NCBI database were very different from each other (Figure 5) (see Figure S8 for other details).

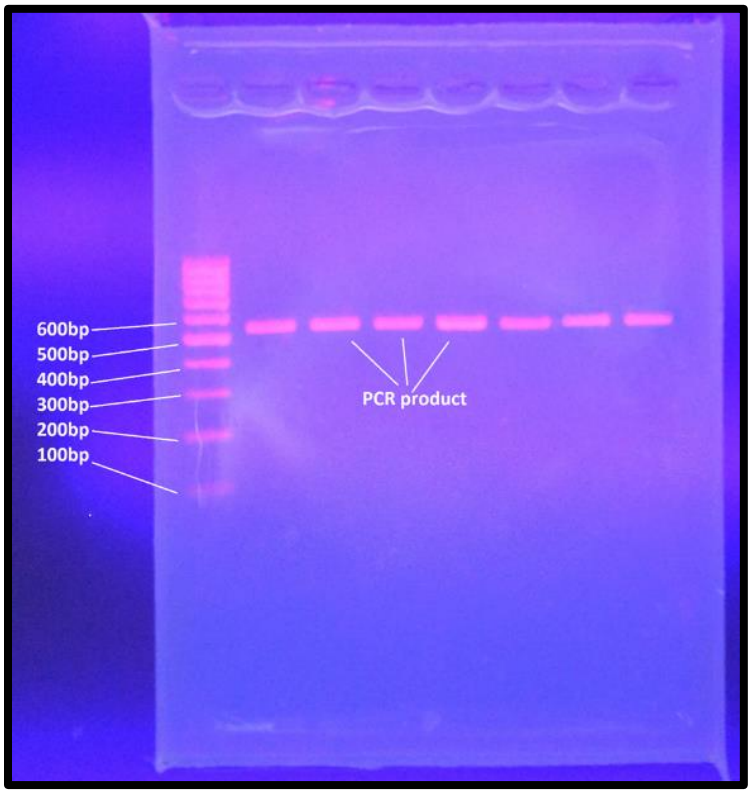

Fig. 4. PCR amplification of the ITS region in seven fungal isolates using agarose electrophoresis. Sanger sequencing was applied to this product.

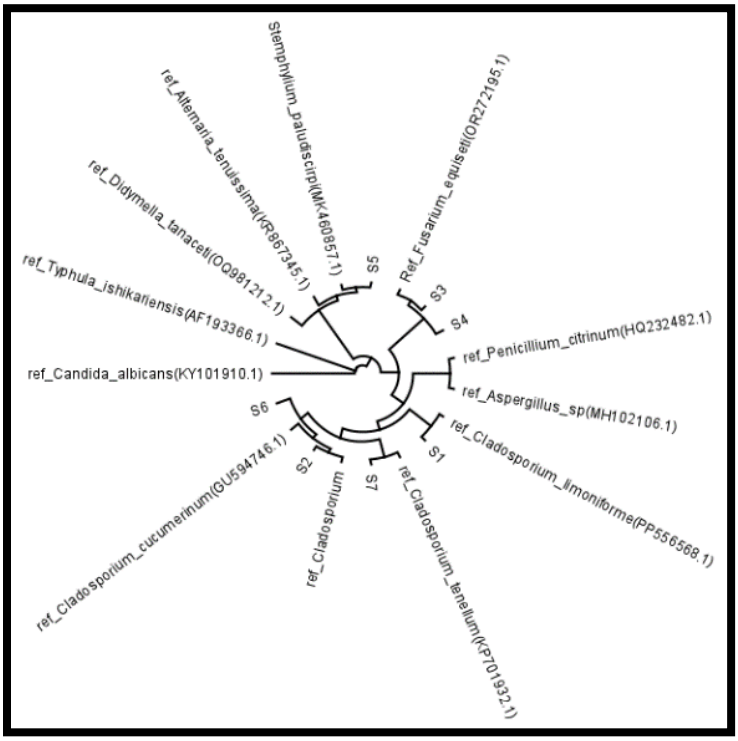

Fig. 5. The whole rectangular evolutionary tree of the intergenic spacer sequences. Each of the previously given figures matched the GenBank accession number of the corresponding organisms. The one that comprehensively tree-categorized at the base of the tree, the number represents the degree of scale range of the creature. The code utilized for the samples under examination is represented by the letter "S#."

DISCUSSION

Human infections from *Cladosporium cladosporioides* are uncommon, yet they have been documented in cases of superficial infection (Castro *et al*., 2013; Annessi *et al*., 1992). These children's positive cultures supported the fungal spp. diagnosis. The two children are in real contact with cats inside the house. Our study findings correspond to previous study (Hamied and Alnedawy, 2024). Out of 130 specimens taken from children who were suspected of having dermatophytes, microscopic analysis revealed 55 specimens with *M. canis* that showed notable variations in the animals' contact. Occasionally, it can result in pulmonary (Kaneda *et al*., 2023) and cutaneous (Matsumoto *et al*., 1994) phaeohyphomycosis (Castro *et al*., 2013; Kantarcioğlu *et al*., 2002). Additionally, it was isolated from brain fluid in a patient with compromised immunological function (Annessi *et al*., 1992; Minnat and Khalaf, 2019). Direct microscopy and specimen culture were used to diagnose the dermatophytes mycologically. Dull green to olive-grey, silky, and tufted colonies grows using a medium of potato dextrose agar (PDA). The colony's feathery borders can range from olive-grey to white. The mycelia rarely climb upward from the colony surface and form mats in dispersed colonies (Bensch *et al*., 2010).

Mature conidiophores resemble trees and are made up of numerous long, branched chains of conidia (Barron, 1968; Campbell *et al*., 2013). The production of *Cladosporium cladosporioides* solitary, unevenly branching conidiophores that range in color from brown to olive-brown, producing numerous consequences (Bensch *et al*., 2010; Barron, 1968). When climbing hyphae reach their terminus, they are conidiophores, which are cylindrical and have thin walls (Bensch *et al*., 2012). The conidia have smooth walls, are lemon-shaped, single-celled, and tiny.

Data showed that for pet cats (33.33%), this is in line with (Jarjees and Issa, 2022), the frequency was higher among home cats (41.8%) compared to outdoor cats (31.8%) in terms of residence. In the children who were analyzed (26.31 %), the prevalence rates considered age, sex, and cat contact. Given that these fungi are opportunistic infections, which usually need a weakened host or a breached skin barrier to develop infection, the low infection incidence in children (5/19) is noteworthy. Females' patients who had weakened or afflicted skin, as well as those who were in proximity to pets, all had higher occurrence rates. There was no statistically significant difference in the overall infection rate between children (26.32%, 5/19) and pet cats (33.33%, 10/30), according to the test results (p-value = 0.749).

According to the analysis, there was no significant difference in the relative abundance of fungal species (such as *Fusarium equiseti*, *Cladosporium spp.*, and *Stemphylium paludiscirpi*) between the groups (p-value = 0.32). For every test, a p-value of less than 0.05 was deemed statistically significant. According to molecular research, a trustworthy technique for identifying fungal species is gene sequencing followed by phylogenetic analysis of the data produced (Boudier, 2001). Hamied and Alnedawy (2024) distinguished between local Iraqi isolates and worldwide isolates of *M. canis* by using the molecular method of diagnosis. A previous study used DNA sequence data analysis to examine a large collection of isolates from clinical specimens in order to evaluate the diversity of *Cladosporium species* linked to human and animal disorders (Sandoval-Denis *et al*., 2015). Since several writers have demonstrated the importance of rRNA for species delimitation in this bacterium (Curtis *et al*., 1994; Zeng *et al*., 2006), we used it to identify *Cladosporium species* in our work.

CONCLUSION

Cladosporium species and *Fusarium* were detected in seven samples of otitis externa in pet cats. The findings of this study have brought significance to the view that pet cats can be an infectious source of this species to children in 26.31 % of patients, and that the majority of children have close experiences with cats within their household, which is commonly indicative of the problematic organism in their pet cats. This study cultured otitis externa samples and examined the variety of *Cladosporium* and *Fusarium species* using molecular characterization of the isolates.

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CONFLICT OF INTEREST

The authors have declared that no competing interests exist.

Supporting information

Fig. 1 S1. The exact position of the retrieved PCR amplicon.

Fig. 2 S1. Nucleic acid sequences alignment.

Fig. 3 S1. Sanger sequence chromatogram.

Table 1 S1. The identified nucleotide sequences of sample 1.

Fig. 1 S2. The exact position of the retrieved PCR amplicon.

Fig. 2 S2. Nucleic acid sequences alignment.

Fig. 3 S2. Sanger sequence chromatogram.

Table 1 S2. The identified nucleotide sequences of sample 2.

Fig. 1 S3. The exact position of the retrieved PCR amplicon.

Fig. 2 S3. Nucleic acid sequences alignment.

Fig. 3 S3. Sanger sequence chromatogram.

Table 1 S3. The identified nucleotide sequences of sample 3.

Fig.1 S4. The exact position of the retrieved PCR amplicon.

Fig. 2 S4. Nucleic acid sequence alignment.

Fig. 3 S4. Sanger sequence chromatogram.

Table 1 S4. The identified nucleotide sequences of sample 4.

Fig. 1 S5. The exact position of the retrieved PCR amplicon.

Fig. 2 S5. Nucleic acid sequences alignment.

Fig. 3 S5. Sanger sequence chromatogram.

Table 1 S5. The identified nucleotide sequences of sample 5.

Fig. 1 S6. The exact position of the retrieved PCR amplicon.

Fig. 2 S6. Nucleic acid sequence alignment.

Fig. 3 S6. Sanger sequence chromatogram.

Table 1 S6. The identified nucleotide sequences of sample 6.

Fig. 1 S7. The exact position of the retrieved PCR amplicon.

Fig. 2 S7. Nucleic acid sequences alignment.

Fig. 3 S7. Sanger sequence chromatogram.

Table 1 S7. The identified nucleotide sequences of sample 7.

Fig. S8. The complete rectangular phylogenetic tree.

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