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Detection of *GLIP* as virulence gene in *Aspergillus fumigatus* Isolates from *Cutaneous canine Aspergillosis*

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Abstract

Background: Canine aspergillosis, an opportunistic fungal infection that can cause severe respiratory and systemic clinical signs in dogs, is primarily caused by *Aspergillus fumigatus*. Because of its progressive character and challenges in early detection, the disease is regarded as a significant veterinary issue. Finding virulence-associated genes that may contribute to the pathogenicity and severity of infection is necessary to improve diagnostic accuracy.

Methods: A total of one hundred oral and pharyngeal swab samples were taken from dogs in Diyala Province, Iraq, who had respiratory symptoms and suspected fungal infections between September 1, 2025, and February 13, 2026. The samples were identified using microscopic traits and macroscopic colony morphology after being cultivated on Sabouraud Dextrose Agar (SDA) under conventional laboratory conditions.

Fourteen isolates of *Aspergillus fumigatus* were chosen for molecular examination. Using a commercial fungal DNA extraction kit, genomic DNA was isolated in accordance with the manufacturer's instructions. The gliP gene (168 bp), a potential virulence-associated gene, was the target of conventional PCR. Under UV light, 1.5% agarose gel electrophoresis was used to examine the amplified PCR products.

Aim: This study's primary goal was to separate *Aspergillus fumigatus* from dog clinical samples and use traditional PCR as a molecular diagnostic method to identify the gliP virulence gene.

Results: 11 out of 14 *Aspergillus fumigatus* isolates (78.6%) had the gliP gene, according to conventional PCR. Positive samples produced a distinct 168 bp amplicon, indicating effective gene amplification. The findings showed a strong relationship between the existence of verified fungal isolates and molecular detection.

Conclusions: In *Aspergillus fumigatus* isolates from dogs, conventional PCR provides a quick, sensitive, and trustworthy molecular technique for identifying the gliP virulence gene. This approach improves understanding of the molecular basis of canine aspergillosis, aids early infection detection, and increases diagnostic accuracy.

Keywords: *Aspergillus fumigatus*, GlIP, PCR, virulence gene, canine aspergillosis

Introduction

The main species of the genus Aspergillus that causes cutaneous aspergillosis, a fungal skin infection in dogs, is thought to be *Aspergillus fumigatus.* Aspergillus niger and Aspergillus flavus are two other species that have been linked to the disease's genesis (Pal, 2007; Dave *et al*., 2015). Both immunocompromised and immunocompetent animals are susceptible to infection. Crucially, cutaneous aspergillosis can occur as a secondary condition as a result of hematogenous dissemination from a primary pulmonary infection, or as a primary infection after traumatic implantation of fungal elements from contaminated environmental sources (Pal & Dave, 2006; Dave *et al*., 2015). Animals that are regularly exposed to soil and organic debris are more susceptible to infection since Aspergillus species are common in the environment. (Pal, 2007; Pal & Dave, 2006; Dave *et al*., 2015). *Aspergillus fumigatus* is a saprotrophic fungus that reproduces asexually by sporulation and thrives on decomposing organic matter in soil. It is acknowledged as one of the most significant animal pathogenic species (Latgé & Chamilos, 2019; Tell *et al*., 2019). This organism is capable of surviving in harsh environmental conditions, including high temperatures exceeding 50°C, commonly found in decomposing plant material (Schoustra *et al*., 2019). The respiratory tract, especially the lower respiratory system, is most frequently linked to *A. fumigatus* infections in dogs (Neumann, 2016). Conidia's small size allows them to enter the upper respiratory tract and nasal passages and go to the pulmonary alveoli, where infection may develop (Salman & Al-Haddad, 2021). Fungal spores can spread hematogenously or systemically in severe cases, affecting various bodily systems in addition to the lungs (König *et al*., 2016). Conidia may germinate and cause infection under the right host and environmental circumstances (Zakaria, 2024).

Despite the fact that *Aspergillus fumigatus* is the species most commonly linked to instances of aspergillosis (Saunders & Van Bree, 2003), diagnosis is still difficult. Traditional diagnostic techniques like culture and microscopy are frequently employed, but they take a lot of time and may not be able to correctly distinguish closely related species (Raja et al., 2017). For the purpose of identifying fungi and identifying genes linked to virulence, molecular methods like polymerase chain reaction (PCR) have been introduced as more sensitive and specific tools. Recent research has connected the virulence of invasive aspergillosis to gliotoxin production (Sugui, 2007). A transcriptional regulator involved in gliotoxin biosynthesis is encoded by the gliZ gene, whereas a nonribosomal peptide synthetase that initiates gliotoxin production is encoded by the gliP gene (Cramer *et al*., 2006). Understanding the pathogenic potential of *A. fumigatus* isolates is thought to be aided by these virulence factors. The lack of information on the gliP virulence gene's presence in clinical isolates of *Aspergillus fumigatus* from dogs led to the creation of the current investigation. The study's goals were as follows:

Materials and Methods

Samples collection and isolation

The study was carried out between September 1, 2025, and February 13, 2026, using 100 samples from German Shepherd dogs (Canis lupus familiars) with skin lesions that were clinically suspected. The research population, which ranged in age from one to five years, included forty males and sixty girls. Private veterinary clinics and the Veterinary Hospital in Khanaqin, Diyala Province, Iraq, provided the samples. Samples were aseptically taken from the affected skin lesions using sterile swabs. All samples were appropriately labeled after collection and sterilely transported to the lab for additional examination.

Isolation and identification of *Aspergillus fumigatus*

Samples were placed onto Sabouraud Dextrose Agar (SDA; Oxoid, UK), which was made by dissolving 65 g of powder in 1 L of distilled water and then autoclaving for 15 minutes at 121°C. Next, the medium was aseptically transferred into sterile Petri dishes.To enable fungal development, the inoculation plates were incubated for five to seven days at 25 to 28°C. Colony morphology, including color, texture, and growth pattern, was documented macroscopically following incubation.Small sections of fungal colonies were put on glass slides and stained with Lactophenol Cotton Blue (LPCB) for microscopic analysis. Using a light microscope, the presence of conidia, hyphae, and conidiophores was noted.The colonies' combined macroscopic and microscopic morphological traits were used to identify *Aspergillus fumigatus.*

Molecular Identification of *Aspergillus fumigatus* using PCR

DNA Extraction:

The DNA of *A. fumigatus* was extracted according to the instructions of mini prepTM kit manufacturer which mentioned as below steps.

1.Sample Preparation: A ZR BashingBeadTM Lysis Tube was filled with 50–100 mg (wet weight) of fungal cells that had been re-suspended in up to 200 µl of sterile water.
2. Lysis: The tube was filled with 750 µl of Lysis Solution.
3. Bead Beating: For five minutes, tubes were treated at full speed in a bead beater.
4. Centrifugation: For one minute, tubes were spun at 10,000 × g.
5. Filtration: Up to 400 µl of the supernatant was put into Zymo-SpinTM IV Spin Filters (Orange Tops) and spun for one minute at 7,000 × g. Note: Prior to usage, the filter base was removed.
6. Binding: Fungal/Bacterial DNA Binding Buffer (1,200 µl) was added to the filtrate.
7. Column Loading: 800 µl of the mixture was put to a Collection Tube Zymo-SpinTM IIC Column, and it was centrifuged for one minute at 10,000 × g. The remainder of the mixture underwent the same process after the flow-through was discarded.
8. Washing: After receiving 200 µl of the DNA Pre-Wash Buffer, the column was centrifuged for one minute at 10,000 × g. Next, it was centrifuged using 500 µl of the Fungal/Bacterial DNA Wash Buffer under the same conditions.
9. Elution: The column was placed in a fresh 1.5 ml microcentrifuge tube, and the column matrix was filled with 100 µl of DNA Elution Buffer. To elute the purified DNA, the centrifuge was set to 10,000 g for 30 seconds.

Purity and concentration of DNA

Genomic DNA was measured for concentration and purity using a NanoDrop spectrophotometer (Nabi, Korea). In summary, 1-2 µL of each DNA sample was analyzed at wavelengths of 230, 260, and 280 nm on the NanoDrop pedestal to determine absorbance. DNA content was measured using absorbance at 260 nm (A260), and DNA purity was evaluated using the A260/A280 ratio. A ratio between 1.8 and 2.0 was assumed to indicate high-quality DNA with minimal protein contamination (Gallagher, 1994).

The Primers' Design and Preparation

We bought gliP gene-targeting primers from Integrated DNA Technologies (IDT, Canada). To get a stock concentration of 100 pmol/µL, these primers were supplied in lyophilized form and then dissolved in nuclease-free distilled water. The stock solutions were stored at -20°C until they were required. In order to prepare working solutions at a concentration of 10 pmol/µL, 10 µL of the stock was diluted with 90 µL of nuclease-free water in sterile conditions.

Table1: Primer Sequences for GLIP Gene

Primer	Sequence (5' → 3')	Product Size
GLIP-F	AAACCCCTGTGAATGCAGACAAAAA	168 bp
GLIP-R	CCCCTTGAGATGAAAGGTGACCCCCCC	

Polymerase Chain Reaction

PCR PreMix Kit

The Maxime PCR PreMix kit (i-Taq, Cat. No. 25025, Intron, Korea) was used for every PCR amplification. The components of a 20 µl reaction mixture (i-Taq DNA Polymerase, dNTPs, reaction buffer, and gel loading buffer) were pre-mixed in each tube. *Aspergillus fumigatus* isolates were used to extract genomic DNA, and the manufacturer's instructions for PCR amplification of the gliP gene were followed (Hays & Ammari, 2025).

PCR Reaction Mixture

The PCR reaction mixture was made for every sample as indicated in Table 2.

Table 2: PCR Reaction Components

Component	Volume (µl)	Final Concentration
Taq PCR PreMix	5	1X
Forward Primer (10 pmol/µl)	1	0.4 pmol/µl
Reverse Primer (10 pmol/µl)	1	0.4 pmol/µl
Template DNA	1.5	Variable
Nuclease-free Water	16.5	-
Total Volume	**25**	

PCR Condition Optimization

The ideal annealing temperature was found using a gradient PCR. Additionally, several DNA template concentrations (1.5-2 µl) were assessed. The ideal conditions for thermal cycling are presented in Table 3.

Table 3: Optimized Thermal Cycling Conditions of GLIP gene

No.	Phase	Temperature (°C)	Time	Cycles
1	Initial Denaturation	95	5 min	1
2	Denaturation	95	40 sec	40
3	Annealing	58	45 sec	40
4	Extension	72	45 sec	40

5	Final Extension	72	5 min	1

Agarose Gel Electrophoresis

The method used for agarose gel electrophoresis was) Sambrook *et al*. 1989). In short, 100 mL of 1X TBE buffer was heated in a microwave until 1.5 g of agarose was completely melted. After cooling to 45 to 50 degrees Celsius, the mixture was poured into a gel casting tray using the proper comb and allowed to set for half an hour at room temperature. 5 µL of PCR product and 3 µL of 6X loading dye (Intron, Korea) were used to load the sample into the gel wells. As a molecular size marker, a 100 bp DNA ladder (Intron, Korea) was employed. The dye front reached about two-thirds of the gel length after one to two hours of electrophoresis in 1X TBE buffer at 5 V/cm. Following electrophoresis, the gel was stained for 30 minutes using RedSafe Nucleic Acid Staining Solution (Intron, Korea) and examined using a UV transilluminator (336 nm; Vilber Lourmat, France). A gel documentation system (Labnet, USA) was used to record images. The excitation and emission wavelengths of RedSafe, a safe fluorescent dye substitute for ethidium bromide, are 309–419 nm and 537 nm, respectively.

Results

Identification of *Aspergillus fumigatus* Isolates

Aspergillus fumigatus was found in 14 (14%) of the 100 samples using the culture technique. Out of 106 samples, 22 Aspergillus niger isolates were found (20.75%), according to(Hadi, Al-Ezzy, & Al-Zuhairi, 2025). When *A. fumigatus* isolates were examined under a microscope, they displayed velvety growth features. At the beginning of their growth, *A. fumigatus* colonies were white, but as they progressed, they acquired characteristic morphological characteristics. A light microscope was used to examine fungus samples under a microscope (Talib and Abdalshaheed, 2026). Figure 2 displays the macroscopic appearance of colonies on Sabouraud Dextrose Agar (SDA), while Figure 1 depicts the microscopic morphology of the fungal cells.

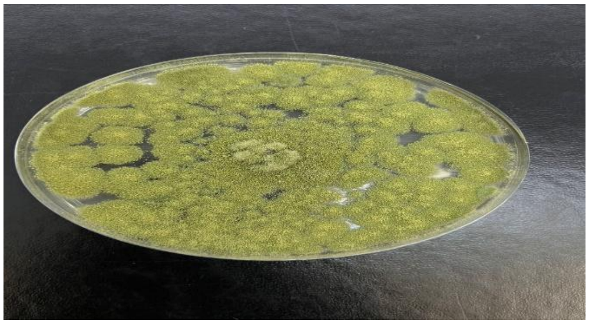

Figure 1: *Aspergillus fumigatus* colonies' macroscopic appearance on SDA after five days at 35°C.

Gel Electrophoresis of Genomic DNA

The band of DNA is displayed in Figure 3.

PCR Amplification of *GLIP* Gene

Using electrophoresis on a 1% agarose gel, the isolated genomic DNA was examined for 30 minutes at 5 V/cm. The PCR results were validated using agarose gel electrophoresis. Of the 14 samples that were examined, 11 (78.6%) had the gliP gene. The amplification yielded the anticipated 168 bp fragment.

Discussion

The current work shows how a molecular method for identifying the gliP gene in pathogens isolated from canine oral and pharyngeal samples has been successfully optimized. The gliP gene was found in 78.6% of the examined isolates, according to the results, suggesting that *Aspergillus fumigatus* strains that colonize these anatomical locations are widely distributed.

From every sample, significant amounts of high-quality genomic DNA were successfully extracted for PCR analysis. According to the results was revealed by (Desjardins and Conklin, 2011), the A260/A280 ratios ranged from 1.71 to 1.99, indicating adequate DNA purity. These results validate the ZR fungus/Bacterial DNA MiniPrep kit's effectiveness in extracting fungus DNA from clinical materials.

The ZR BashingBeadTM Lysis Tubes with 0.1 mm and 0.5 mm beads efficiently enabled fungal cell walls to be mechanically disrupted. According to (Fredricks *et al*. 2005), the fungal cell wall is recognized as a significant obstacle to effective DNA extraction processes. In this investigation, full cell lysis and DNA release were achieved with a 5-minute bead-beating stage at maximal speed with lysis buffer. The best annealing temperature for PCR optimization was discovered to be 58°C for gliP gene amplification. Despite the fact that the theoretical melting temperatures (Tm) varied from 60.1°C to 71.0°C, this outcome is in line with the findings of (Rychlik *et al*.1990), who stated that reaction conditions, primer-template interactions, and template complexity frequently result in optimal annealing temperatures being lower than predicted Tm values. For diverse clinical fungal isolates in particular, gradient PCR was crucial to obtaining the best specificity and amplification efficiency. The gliP gene was found in 78.6% of isolates, indicating that it plays a significant role in the pathogenicity and survival of *Aspergillus fumigatus* in the oral and pharyngeal environments of dogs. Genetic variation or mutations influencing primer binding sites may be the cause of some isolates' lack of amplification, even though DNA quality was verified by electrophoresis and spectrophotometry. GDSL lipase enzymes involved in fungal development, stress adaptability, and virulence are encoded by the gliP gene. These enzymes increase fungal pathogenicity by aiding in tissue invasion and nutrition uptake. GliP proteins contribute significantly to fungal infection by promoting host tissue colonization and survival, according to (Kwon Chung & Sugui 2009).

DNA imaging was made safer and more efficient by using Red Safe Nucleic Acid Staining Solution rather than ethidium bromide (Khalili *et al*., 2015). Red Safe is less mutagenic and fluoresces clearly when exposed to UV light, making it possible to detect PCR products accurately.

The protocol used in this study has a number of benefits, such as quick processing time (about 4 hours from DNA extraction to results), high specificity because of optimized annealing conditions, use of non-carcinogenic staining reagents, and suitability for a variety of clinical sample types.

Conclusions

In canine oral cavity and pharyngeal swab samples, fungal targeting identified the gliP gene as a frequent genetic marker. Particularly at an annealing temperature of 58°C, the improved PCR technique for this gene was very specific and dependable.

Recommendations

To more accurately determine the distribution of the gliP gene in canine populations and the incidence of *Aspergillus fumigatus*, future research is advised to use a greater number of samples gathered from various geographic locations. To increase diagnostic precision and offer a more thorough genetic characterisation of fungal isolates, multiplex PCR that targets several virulence-associated genes is also recommended. Additionally, more research is required to assess the connection between the gliP gene's existence and the pathogenicity and clinical severity of canine aspergillosis. Lastly, for the quick and accurate identification of fungal pathogens, the use of PCR-based molecular diagnostic tools in routine veterinary laboratory practice is advised.

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Ethical Approval

This study received ethical approval from the University of Diyala's College of Veterinary Medicine. The institutional rules for the care and use of animals in research were followed in all animal-related procedures.

Conflict of Interest

Regarding the publishing of this paper, the authors state that they have no conflicts of interest.

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Author Contributions

H.A.A. made contributions to data analysis, laboratory work, sample collection, study design, and manuscript writing. A.M.A. helped with the study's final approval, critical manuscript review, and supervision.

References

Pal, M. (2007). *Veterinary and medical mycology* (1st ed.). Indian Council of Agricultural Research.

Dave, P., Mahendra, R., & Pal, M. (2015). Etiologic significance of *Aspergillus terreus* in primary cutaneous mycosis of an agricultural worker. *Molecular Microbiology Research, 5*(2), 1–4.DOI: 10.5376/mmr.2015.05.0002

Pal, M., & Dave, P. (2006). Aspergillosis: A sapromycotic zoonosis. *Intas Polivet, 7*, 421–428.

Latgé, J.-P., & Chamilos, G. (2019). *Aspergillus fumigatus and aspergillosis in 2019. Clinical Microbiology Reviews, 33*(1), e00140-18.https://doi.org/10.5376/mmr.2015.05.0002

Tell, L. A., Burco, J. D., Woods, L., & Clemons, K. V. (2019). Aspergillosis in birds and mammals: Considerations for veterinary medicine. In *Recent developments in fungal diseases of laboratory animals* (pp. 49–72). Springer.
DOI: 10.1007/978-3-030-18586-2_4

Schoustra, S. E., Debets, A. J. M., Rijs, A. J. M. M., Zhang, J., Snelders, E., Leendertse, P. C., … Verweij, P. E. (2019). Environmental hotspots for azole resistance selection of *Aspergillus fumigatus*, the Netherlands. *Emerging Infectious Diseases, 25*(7), 1347–1353.doi: 10.3201/eid2507.181625

Neumann, N. (2016). Aspergillosis in domesticated birds. *Journal of Comparative Pathology*. DOI: 10.1016/j.jcpa.2016.07.003

Salman, R. A., & Al-Haddad, Z. A. (2021). Isolation and identification of *Aspergillus* spp. from human and sheep respiratory infection in Al-Qadisiyah Province. *Systematic Reviews in Pharmacy, 12*(3), 1–6.

König, H. E., Navarro, M., Zengerling, G., & Korbel, R. (2016). Respiratory system (apparatus respiratorius). In H. E. König et al. (Eds.), *Avian anatomy textbook and colour atlas* (2nd ed., pp. 118–130). 5M Publishing.https://www.worldcat.org/search?q=9781910455609

Zakaria, L. (2024). An overview of *Aspergillus* species associated with plant diseases. *Pathogens, 13*(9), 813. https://doi.org/10.3390/pathogens13090813

Saunders, J. H., & Van Bree, P. (2003). Diagnosis of nasal aspergillosis in the dog. *Vlaams Diergeneeskundig Tijdschrift, 72*, 399–408.https://doi.org/10.21825/vdt.89254

Raja, H. A., Miller, A. N., Pearce, C. J., & Oberlies, N. H. (2017). Fungal identification using molecular tools: A primer for the natural products research community. *Journal of Natural Products, 80*(3), 756–770. DOI: 10.1021/acs.jnatprod.6b01085

Sugui, J. A., Pardo, J., Chang, Y. C., et al. (2007). Gliotoxin is a virulence factor of *Aspergillus fumigatus*: *gliP* deletion attenuates virulence in mice. *Eukaryotic Cell*, *6*(9), 1562–1569.DOI: 10.1128/EC.00141-07

Cramer, R. A., et al. (2006). Disruption of a nonribosomal peptide synthetase in *Aspergillus fumigatus* eliminates gliotoxin production. *Eukaryotic Cell*, *5*(6), 979–983.DOI: 10.1128/EC.00049-06

Gallagher, S. R. (1994). Quantitation of DNA and RNA with absorption and fluorescence spectroscopy. *Current Protocols in Human Genetics, A.3D*, 1–8.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). Cold Spring Harbor Laboratory Press.

Desjardins, P. R., & Conklin, D. S. (2011). Microvolume quantitation of nucleic acids. *Current Protocols in Human Genetics, 93*(1), A.3J.1–A.3J.16. **DOI:10.3791/2565**

Fredricks, D. N., Smith, C., & Meier, A. (2005). Comparison of bead-beating and chemical lysis methods for fungal DNA extraction from clinical specimens.DOI: 10.1128/JCM.43.10.5122-5128.2005

Rychlik, W., Spencer, W. J., & Rhoads, R. E. (1990). Optimization of annealing temperature in PCR using gradient thermocycling. *Nucleic Acids Research, 18*(21), 6409–6412.

Kwon-Chung, K. J., & Sugui, J. A. (2009). What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*? *Medical Mycology, 47*(S1), S97–S103. DOI: 10.1080/13693780802056012

Khalili, E., Hosseini, V., Solhi, R., & Aminian, M. (2015). Rapid fluorometric quantification of bacterial cells using RedSafe nucleic acid stain. *Iranian Journal of Microbiology*, *7*(6), 319–323. https://pmc.ncbi.nlm.nih.gov/articles/PMC4752686

Talib, N., & Abdalshaheed, D. (2026). Gliotoxin production from *Aspergillus fumigatus* isolated from bovine respiratory infection. *Diyala Journal for Veterinary Sciences*, *3*, 50–62. . https://doi.org/10.71375/djvs.2025.03404

Hays, A. K., & Ammari, A. M. (2025). Antimicrobial effects of capsaicin extracted from *Capsicum annuum* on *HWP1* gene expression of *Candida albicans*. *Diyala Journal for Veterinary Sciences*, *3*(2), 124–132. https://doi.org/10.71375/djvs.2025.03210

Hadi, R. M., Al-Ezzy, A. I., & Al-Zuhairi, A. H. K. (2025). Phenotypic identification and molecular characterization of *Aspergillus niger*, fumonisin and ochratoxin genes from otomycosis in domestic cats. *Diyala Journal for Veterinary Sciences*, *3*(1), 120–131.