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Molecular Identification and Virulence Gene Profiling of *Aspergillus fumigatus* Isolated from Feline Respiratory Infections

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***Abstract*—** Respiratory aspergillosis in felines is major problems in diagnosis and treatment, thereby leading to a high rate of morbidity. The reason for the pathogenic success of *Aspergillus fumigatus* lies in the presence of certain through virulence factors, which enable the pathogen to invade the host tissue and evade the host immune system. The study aimed to identify the *Aspergillus fumigates* isolate from respiratory infections of cats by PCR of the mitochondrial ND2 gene and virulence genes with sequencing. PCR amplification of the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene was used to verify the presence of A. *fumigatus*. The virulence potential of the confirmed isolates was assessed by the presence of the alkaline protease (alp1) and aspartic protease (asp) genes. The molecular study showed that the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene was used to verify the presence of A. *fumigatus* in 35 isolates from totally 41 phenotypically characterized isolates during six months of sample collection period in different veterinary medicine clinics and centers in Karbala province, also the study showed that the a considerable difference in the occurrence of important protease gene sequences among the 35 feline A. fumigatus isolates, the alp1 gene that encodes alkaline protease was present in 100% (all 35) of the isolates, emphasizing the importance of this gene in the overall pathogenicity of this fungus.

However, the asp gene that encodes aspartic protease was present in only 15 (42.9%) of the isolates. DNA sequencing was performed on the isolates and give accession numbers LC915276, LC915275 and LC915273 for Mitochondrial NADH subunit 2(ND2), Alkaline protease (alp1) and Aspartic protease (asp) respectively. The sequences were then submitted to the NCBI GenBank database to ensure that a genetic fingerprint existed in the public domain.

These findings highlight on the importance of molecular diagnosis of feline aspergillosis and reveal important virulence gene repertoire, which suggests that although alp1 is a key gene, asp might play a role in the pathogenicity of a subset of A. *fumigatus* strains in cats.

***Keywords* —** *Aspergillus fumigatus*, Mitochondrial DNA, Respiratory Infections, Protease Virulence Genes.

INTRODUCTION

The mitochondrial DNA (mtDNA) of Aspergillus fumigatus is a compact, circular genome that has a faster rate of evolution than single-copy nuclear genes, which allows the acquisition of polymorphisms that are useful for discriminating between strains. The (mt) genome is known to have a high copy number per cell, especially when dealing with clinical samples, where fungal biomass is often limited or degraded (1). This high cell count in the cell makes mitochondrial DNA better target for diagnostic tests to identify pathogens in a patient's sample, avoiding false-negative results.

In the mitochondrial genome, the genes that encode the subunits of the NADH dehydrogenase complex, such as the NADH dehydrogenase subunit (ND2 gene), are identified as potential candidates for the detection and differentiation of fungi. In this context, the ND2 gene is of great importance in the context of oxidative phosphorylation, and like other protein-encoding regions of the mitochondrion, this gene has a higher rate of mutations compared to the ribosomal regions of the nuclei, which is a key factor in analyzing the population of A. fumigatus (2) Moreover, the ND2 gene is an extremely potent

molecular marker, which can easily be amplified and potentially provide a very in-depth understanding of the phylogenetic strain population. (3).

Aspergillus fumigatus synthesizes the major virulence factor, an alkaline protease (alp1). This enzyme is encoded by the alp1 gene. This enzyme is a subtilisin-like serine protease with maximum activity at an alkaline pH, which is usually encountered an inflammatory response. The synthesis and secretion of this enzyme have been found to be highly induced during the conidia germination and the invasive hyphal development phase of the A. fumigatus life cycle, during which the pathogenic phase of this fungus occurs. It is assumed that the major role of this enzyme is to degrade the structural proteins of the host, which helps the advancing fungus to obtain nutrients (4,5).

The virulent effect of alkaline protease is achieved through its highly potent activity on elastin and collagen. This protease acts on the integrity of the tissues of the host by degrading essential components of the extracellular matrix, including collagen and elastin. This degradation provides a pathway for the entry of the hyphal form and also affects the defense mechanism of the tissues. This can lead to the unmasking of concealed adhesin-binding sites and the impairment of the functioning of the cells of the epithelium and endothelium (6). Direct in vitro studies have demonstrated the degradation of essential proteins of the extracellular matrix, including laminin and fibronectin, which play a critical role in the adhesion of cells (7) Furthermore, the degradation of proteins can lead to the production of peptides, which can act as a nutrient source for the pathogen and thus facilitate its growth in the nutrient-poor medium.

The alkaline protease of this pathogen also exhibits potent immunomodulatory activities in addition to its direct tissue damage activities by destruction of the membrane attack complex and the process of opsonization. Consequently, this leads to reduced complement-mediated killing of the fungal components. The proteolytic inactivation of critical and immunological mediators' roles in immune cells recruitment to the site of infection. For example, the immunomodulation of IL-6 and other pro-inflammatory cytokines by this enzyme has been proposed to underlie the mechanism of action through which this enzyme reduces host inflammation and facilitates a permissive environment for the colonization of this pathogen (8).

Additionally, this enzyme can interfere with host immunoglobulins and modify the activities of cytokines and chemokines. This results in the inhibition of the adaptive immune response (9). alp1 activation may be regulated through the central pH-responsive transcription factor PACC, which senses environmental pH changes to activate expression of the gene under alkaline conditions specific to certain host regions (10,11). Moreover, the nitrogen catabolite repression pathway ensures the activation of the enzyme when preferred nitrogen sources become depleted, and the fungus must utilize host-derived proteins, thereby directly relating nutrient sensing to virulence (12).

Aspartic protease is encoded by the asp gene, its main function is to assist the fungus in the invasion of the host tissue and the acquisition of nutrients through the degradation of the host's main structural proteins. This protease, being an extracellular enzyme, has the best activity in the hydrolysis of macromolecular structures such as collagen, elastin, and fibrinogen, which are the main structural components of the lung tissue's extracellular matrix and clots, respectively (13). Through the hydrolysis of the host's structural proteins, the protease makes the invasion of the host's tissue by the fungal hyphae easier, thereby causing significant damage to the host's tissue .In addition, the protease's hydrolyzing activity on the host's structural proteins not only makes the physical invasion of the host's tissue easier, but the hydrolyzing products, such as bioactive peptides, also cause the host's tissue to become inflamed, thereby providing the fungus with the nutrients that it needs to grow. In this regard, the hydrolyzing activity of the protease on the host's structural proteins provides the fungus with the nutrients that it needs to grow and develop, despite the nutrient-scarce environment (14).

In addition to its role in the hydrolysis of peptides, aspartic acid protease is also involved in the disruption of the host's immune response. The enzyme can directly interfere with the host's innate immune response by degrading critical antimicrobial peptides and proteins. The enzyme, in particular, can disintegrate and inactivate critical opsonin and complement proteins, thereby disrupting the host's ability to mark the pathogenic fungal cells for phagocytosis by immune cells, including neutrophils and macrophages (9). This disruption of the host's immune response is an important mechanism of immune evasion by pathogenic fungi.

The aim of the current study, is phenotypically and molecular identification of the Aspergillus fumigatus isolated from respiratory infections of cats by PCR amplification of the mitochondrial ND2 gene, and two key virulence genes (alkaline protease (alp1 gene) and aspartic protease (asp gene), and then sequencing the isolates.

MATERIALS AND MTHODS

1. Isolation of Aspergillus fumigatus from cat

The swabs of respiratory tract were cultured on Caspeck's agar and incubated at

37°C for 5 days to ensure good sporulation of A. fumigatus.

2. Genomic DNA extraction

The DNA was extracted from identified A. fumigatus isolates using a Zymo Research Fungal/Bacterial DNA MiniPrep™ Kit (USA).

3. Estimation of the DNA concentration and purity The concentration of DNA was detected using the Nanodrop. The optical density (O.D) was determined using the Nanodrop at 260/280nm ratio using 1.5 µl of the extracted DNA. (15).

4. Preparation of Primers Stocks Every primer (ND2, alp1 and asp) in Table (1) were designed based on the Prim3Plus program. These primers were supplied by Macrogen Biotech company in a lyophilized form, these primers were diluted in nuclease-free water, resulting in a final concentration of 100 picomoles/µl, and stored in the deep freezer at -20C0 until the PCR amplification was carried out. For the reaction, a work solution was prepared by adding 10 µl of the primer stock solution and 90 µl of nuclease-free water, resulting in a final concentration of 10 picomoles/µl.

Table 1. Primers that used in this study

Primers		5 – sequence-3	Amplicon size (bp)
dehydrogenase subunit 2 (ND2)	F	5-TGGTATTGGGTTATTTGGTGGA-3	522 bp
	R	5-TCTTGTTCATCAACTATACCTGCT-3	
Aspartic Protease (asp)	F	5-TGGACATGCATCAACCAA-3	315 bp
	R	5-GTCAAACTTATAGTCGTG-3	
Alkaline protease (alp1)	F	5-AGCACCGACTACATCTAC-3	747 bp
	R	5-GAGATGGTGTTGGTGGC-3	

5. Conventional PCR Amplification

Polymerase Chain Reaction (PCR) was carried out for the amplification of the target genes. The reaction mix was prepared for each gene with a final volume of 25 µL (Table 2). The temperature and cycles of the PCR amplification programs were showed in Table 3.

Table 2. Reaction components of PCR

NO.	Component	25 µL (final volume)
1	Dream Taq Green PCR Master Mix	12.5 µL
2	Forward primer	1 µL
3	Reverse primer	1 µL
4	DNA	3 µL
5	Nuclease-free water	7.5 µL

Table 3. Amplification program of primers

No	Amplified gene	Initial denaturation	No. of cycle	Denaturation	Annealing	Elongation	Final extension
1	**ND2**	95°C/ 5min	35	94°C/ 45 sec	57°C/ 35sec	72°C/ 1min	72°C/ 5min
2	**asp**	95°C/ 5min	35	94°C/ 45 sec	54°C/ 35sec	72°C/ 1min	72°C/ 5min
3	**alp1**	95°C/ 5min	35	94°C/ 45 sec	56°C 35sec	72°C/ 1min	72°C/ 5min

6. Agarose gel electrophoresis

Gel Electrophoresis was used for the detection of amplified products, the amplified products of the PCR reaction were visualized with the help of Ethidium bromide dye and UV transilluminator documentation. (16)

7. Sequencing of PCR Amplicons

The PCR products that have been successfully amplified were subjected to sequencing with the help of an automated DNA sequencing machine supplied by Macrogen Corporation, Erbil. The same primer was used for the sequencing reaction. The sequence was analyzed using BioEdit and MEGA11(USA).

8. Interpretation of sequencing data

The sequencing results obtained from the PCR products were used to compare them with their corresponding sequences in the reference database using BioEdit Sequence Alignment Editor Software Version 7.1 (DNASTAR, Madison, WI, USA). The sequences obtained were used to analyze them and to determine if there were any variations or similarities. Each of the nucleic acid sequences was given a unique identifier depending on their position in the PCR products and the reference genome, Using NCBI-BLAST. was used to obtain specific target regions, which helped in the for identification of the sequences obtained from the PCR products.

9.Ethical Approval: Ethical permission to conduct the research was obtained from the local animal care and use committee at the College of Veterinary Medicine, University of Baghdad according to the number and date (P-G/346: 11/2/2026).

RESULT AND DISCUSSION

The phenotypic distribution of the infected cats

One hundred fifty samples of nasal swabs were collected from suspected respiratory infected cats and

the phenotypic distribution of these cat as shown in (Table 4).

Table 4. The phenotypic distribution of infected cats

Cat breed	Positive %	Negative %	Total
Shirazi	11(26.1%)	31(73.8%)	42
Himalayan	10(26.3%)	28(73.6%)	38
Local	18(32.1%)	38(67.9%)	56
British	2(14.2%)	12(85.7%)	14
Total	41(27.3%)	109 (72.6%)	150

These samples were analyzed, from which *A. fumigatus* was phenotypically identified in 41 cases as shown in Table 4.1, yielding an overall prevalence of 27.3% within the studied population.

A notable variation in the distribution of the fungus was observed across different cat breeds. The highest rate of positive identification was observed in the local breed cats, where 32.1% (18 out of 50) of the samples were found to be positive. In contrast, the lowest positivity rate was recorded in the British breed, with only 14.2% (2 out of 14) of samples testing positive. Shirazi and Himalayan strains showed an intermediary and almost equal percentage of positivity at 26.1% and 26.3%, respectively. This implies that the strain is a determinant of *A. fumigatus* colonization or infection, with local strains having a comparatively higher incidence rate. This study supports previous research which suggests that genetic and environmental factors affect the prevalence of respiratory fungal pathogens among different species. (17). In addition, the presence of differences in prevalence according to breeds is supported by earlier studies regarding feline aspergillosis in which the risk of infection was higher among brachycephalic breeds such as Shirazi and Himalayan breeds, although according to the current study, being a local breed can be another risk factor for contracting the disease (18). High prevalence among local breeds may be related to genetic factors or higher exposure to Aspergillus spores since the outdoors was considered a risk factor for the disease in endemic regions (19).

Molecular Identification of *A. fumigatus*
Molecular analysis of *virulence* genes
Molecular analysis of NADH dehydrogenase subunit 2 (ND2gene).

This choice was informed by the high abundance of the mitochondrial genome within each cell, which enhanced the sensitivity of detection using PCR. The disparity noted between the phenotypic (41 isolates) and molecular methods (35 isolates) is a testament to the risks associated with misdiagnosis when using conventional cultures alone (Figure 1). In recent years, research in the area of mitochondrial genomics has confirmed the importance of ND2 gene subunits as crucial building blocks for next-generation panels aimed at reaching an accurate etiological diagnosis, effective antifungal treatment, and an understanding of the actual prevalence of pathogenic species like *A. fumigatus* (2,20)

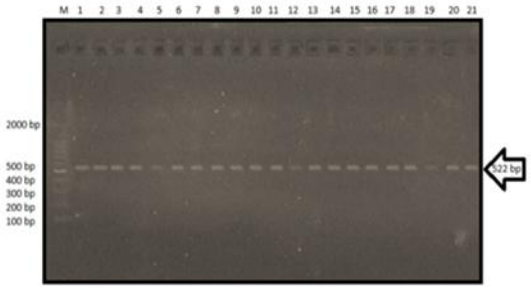

Figure 1. Agarose gel electrophoresis (1.5%) shows bands of the NADH dehydrogenase subunit 2 (ND2gene) of *A. fumigatus* partially amplified (522bp) by the PCR assay. Lane M: DNA ladder (100–2000 bp). Electrophoresis was done at 100 V for 1 hour

Molecular detection of alp1 gene

In this regard, the significant contribution of alkaline protease (alp1) to the disease development in *A. fumigatus* was highly validated by the genetic characteristics of this study. In this experiment, the alp1 gene was found in all *A. fumigatus* specimens (35/35 isolates), giving a positivity rate of 100% (Figure 2). It should be noted that the detection of alp1 in all clinical isolates is vital because this indicates the essential nature of this virulence factor in causing respiratory infections in cats.

The breakdown of the host's extracellular matrix proteins mediated by the alp1 gene is vital to hyphal invasion of tissue. The presence of the gene in all isolates indicates that production of such an enzyme is quite common in this fungal species. If that were not the case, the success of both colonizing and infecting would be greatly compromised (4). Moreover, a positive result recorded in all 43 isolates offers a robust genetic foundation for creating more accurate and targeted diagnosis. As far as PCR analysis of clinical samples is concerned, one might consider the alp1 gene to be a highly reliable genetic biomarker. It confirms findings of previous studies showing that virulence factor genes could be used as targets in PCR diagnostics rather than universal genes (8.21).

Also, the presence of this gene holds great significance in studying host-pathogen interaction studies in feline models. It can be hypothesized that the pathogenesis of feline aspergillosis is greatly influenced by the capability of alp1 gene to cause tissue damage. This observation is supported by the clinical manifestation of the disease, which is associated with destruction of nasal and sinus tissues (22).

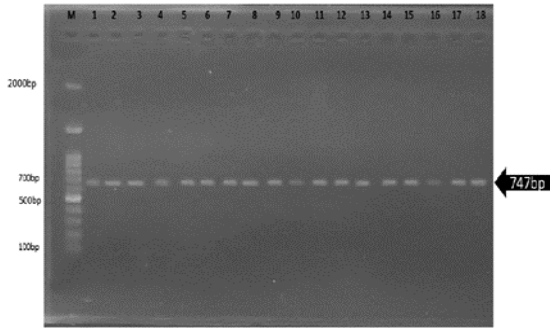

Figure 2. Agarose gel electrophoresis (1.5%) shows bands of the alkaline protease (alp1 gene) of *A. fumigatus* partially amplified (747bp) by PCR assay. Lane M: DNA ladder (100–2000 bp). Electrophoresis was done at 100 V for 1 hour.

Molecular detection of asp gene

The primary finding of this study was that 15 of 35 (42.9%) clinical *A. fumigatus* isolates were positive to this gene (Figure 3). These results are consistent with the emerging understanding that pathogenic fungi exhibit considerable strain-to-strain diversity, with not all clinical isolates sharing an identical virulence gene repertoire. thus, *A. fumigatus* isolates in the cat population may be more dependent on aspartic proteases for host colonization and tissue invasion (4).

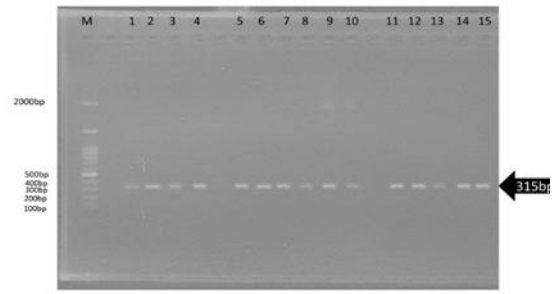

Figure 3. Agarose gel electrophoresis (1.5%) shows bands of the of aspartic protease (asp gene) of *A. fumigatus* partially amplified (315bp) by PCR assay. Lane M: DNA ladder (100–2000 bp). Electrophoresis conditions: 100 V, 1 hour.

The importance of aspartic protease genes, particularly in opportunistic fungal pathogens, has been extensively demonstrated. Among these, the *asp* gene family in *Aspergillus fumigatus* is considered to be of significant value as a molecular target. Aspartate proteases are known to show specificity for aromatic amino acids such as phenylalanine, tyrosine, and tryptophan on both sides of the peptide bond, a property that has been exploited for their identification and functional characterization (22). In other fungal pathogens, the role of aspartic proteases in virulence has been well established. For instance, an aspartic protease (Pep1p) was identified in *Cryptococcus neoformans*, and it was found that improved outcomes were observed in susceptible mice when antibodies against Pep1p were produced (23). Likewise, the secreted aspartic proteases of *Candida* spp. have been shown to be important for multiple virulence-related processes, including biofilm formation and epithelial cell invasion (24). In the context of *A. fumigatus*, the aspartic protease encoded by the *ASP* gene is considered to play an equally critical role. The release of such broad-spectrum proteases by *A. fumigatus* into host tissues can lead to the disruption of immune responses, which is achieved by the digestion of complement system components and the subsequent elicitation of inflammatory responses (25).

The molecular distribution of infected cats

The presence of *A. fumigatus* in respiratory infections in cats was investigated through molecular analysis. Of the 41 phenotypically identified *A. fumigatus* samples, molecular confirmation was achieved in 35 cases by targeting a specific mitochondrial gene region (ND2 gene) as shown in table 5, representing 23.3% of the total. the local breed cats again exhibited the highest prevalence, with 26.8% (15 out of 50) of samples testing positive molecular a notably higher than the phenotypic result. Similarly, the prevalence in the Himalayan breed was adjusted downward from 26.3% phenotypically to 21% by molecular detection. This variation underscores the necessity of using molecular techniques for accurate species identification, as phenotypic methods can sometimes be inconclusive due to morphological similarities between different fungal species (26,27).

The overestimation of prevalence by phenotypic methods observed in this study is a well-documented phenomenon, with molecular methods frequently providing revised estimates due to their superior specificity (28). The high prevalence in local breeds, confirmed by molecular detection, this finding indicates that a greater susceptibility to fungal respiratory infection. It has been hypothesized that this increased susceptibility could be attributed to underlying breed-specific risk factors. Among the factors that have been suggested as critical determinants in the pathogenesis of feline aspergillosis are variations in immune function and differential environmental exposure (29). It is theorized that local cats might possess distinct genetic traits that influence the effectiveness of their pulmonary immune responses, potentially reducing their capacity to clear inhaled fungal spores (30). Furthermore, it is considered plausible that local breed cats are subjected to a greater environmental load of Aspergillus

conidia, potentially due to lifestyle factors such as increased outdoor access, which has been identified as a significant source of exposure (31).

Additionally, the discrepancy between phenotypic and molecular results in the Himalayan and Others breeds highlights the particular value of molecular diagnostics in epidemiological studies, as misidentification can lead to inaccurate prevalence estimates and obscure true risk factor associations (32,33).

Table 5. The molecular distribution of infected cats

Cat breed	Positive %	Negative%	Total
Shirazi	11(26.1%)	31(73.9%)	42
Himalayan	8(21%)	30(79%)	38
Local	15(26.8%)	41(73.2%)	56
British	1(7.1%)	13(92.9%)	14
Total	35 (23.3%)	115 (76.7%)	150

Sequences Analysis

The sequence analysis confirmed the identification genes of *A. fumigatus* (mitochondrial ND2, alp1 and asp) which reported in GenBank NCBI as showed in Table 6.

Table 6. sequence analysis of *A. fumigatus* genes

NO.	Genes	Accession Number	Appendix
1.	mitochondrial ND2 gene	LC915276	Appendix 1
2.	Alkaline protease (alp1gene)	LC915275	Appendix 2
3.	Aspartic protease (asp gene)	LC915273	Appendix 3

The article was concluded a phenotypic prevalence of 27.3% (41 out of 150 samples) was recorded for *A. fumigatus* in respiratory-infected cats, with local breed cats showing the highest susceptibility (32.1%). However, molecular confirmation using mitochondrial NADH dehydrogenase subunit (ND2 gene) reduced the prevalence to 23.3% (35 cases), indicating that phenotypic methods may overestimate infection rates due to misidentification. Addition, the alkaline protease gene (alp1) was detected in 100% of molecular confirmed *A. fumigatus* isolates, underscoring its essential and conserved role as a virulence factor in feline aspergillosis. In contrast, the aspartic protease gene (asp) was detected in only 42.9% of isolates, suggesting strain-to-strain diversity in the virulence gene repertoire among clinical isolates. finally, the identity of all three target genes—mitochondrial NADH dehydrogenase subunit 2(ND2), alkaline protease (alp1), and aspartic protease (asp)—was confirmed by sequence analysis and deposited in the NCBI GenBank database under accession numbers LC915276, LC915275 and LC915273respectively.

CONCLUSION

This study concluded how crucial is the molecular diagnosis of cat aspergillosis and also indicate an important set of virulence genes, which imply that even though the alp1 gene is very crucial, asp could possibly be involved in the pathogenicity of some isolates of A. fumigatus in cats.

AI Declaration

We responsible for the accuracy and integrity of all content of the manuscript, including part generated by AI, and It is not used as a co-author.

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Conflict of interest

The Authors declare that there is no conflict of interest.

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Nucleotide — Nucleotide — Advanced

GenBank — Send to:

Aspergillus fumigatus IQ1 mitochondrial ND2 gene for NADH dehydrogenase subunit 2, partial cds

GenBank: LC915276.1
FASTA Graphics

Go to:

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2026
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            Aspergillus; Aspergillus subgen. Fumigati.
REFERENCE   1
  AUTHORS   Hameed,M.A.K., Huda,S.J.A.B. and Sabah,Zk.
  TITLE     Aspergillus fumigatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 277)
  AUTHORS   Hameed,M.A.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JAN-2026) Contact:Mustafa Abdul Kareem Hameed
College
            of Veterinary Medicine, Microbiology; Karbala 65001, Iraq
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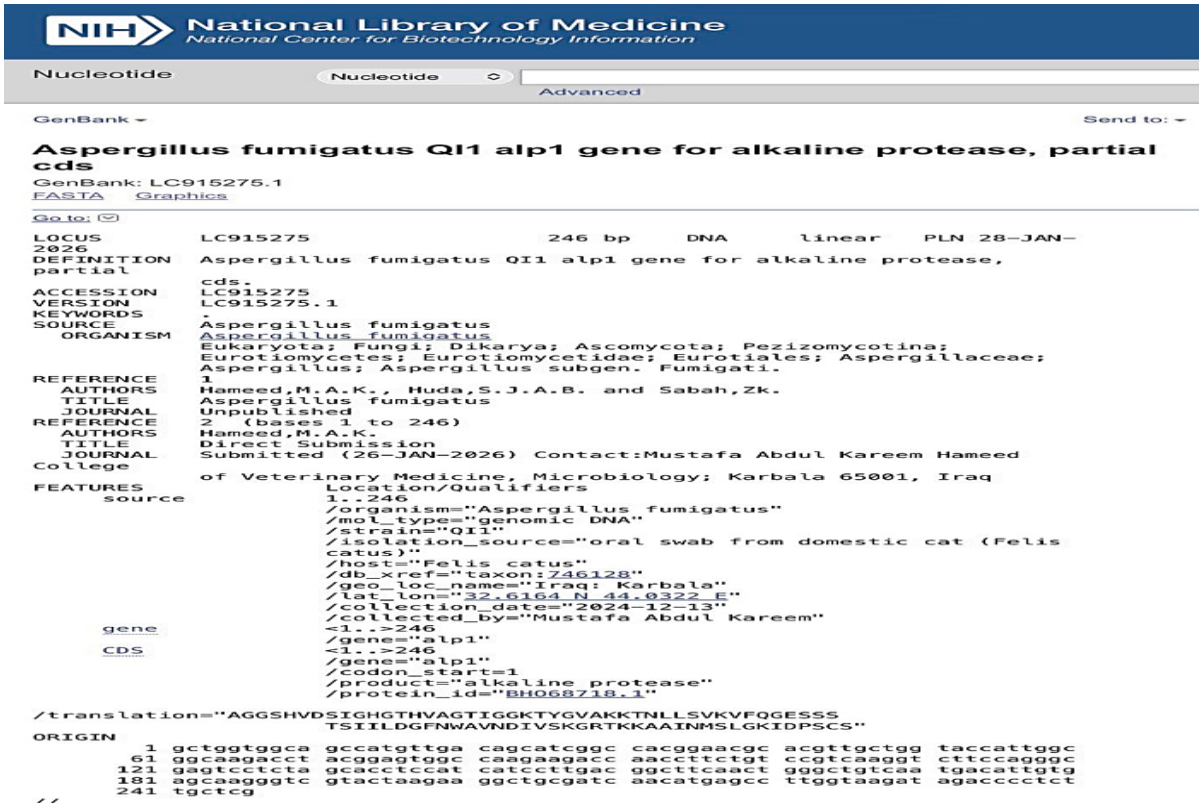
Appendix 1: Registration of mitochondrial ND2 gene of *A. fumigatus* within GenBank /NCBI



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Nucleotide — Nucleotide — Advanced

GenBank — Send to:

Aspergillus fumigatus QI1 alp1 gene for alkaline protease, partial cds

GenBank: LC915275.1
FASTA Graphics

Go to:

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2026
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            Aspergillus; Aspergillus subgen. Fumigati.
REFERENCE   1
  AUTHORS   Hameed,M.A.K., Huda,S.J.A.B. and Sabah,Zk.
  TITLE     Aspergillus fumigatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 246)
  AUTHORS   Hameed,M.A.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JAN-2026) Contact:Mustafa Abdul Kareem Hameed
College
            of Veterinary Medicine, Microbiology; Karbala 65001, Iraq
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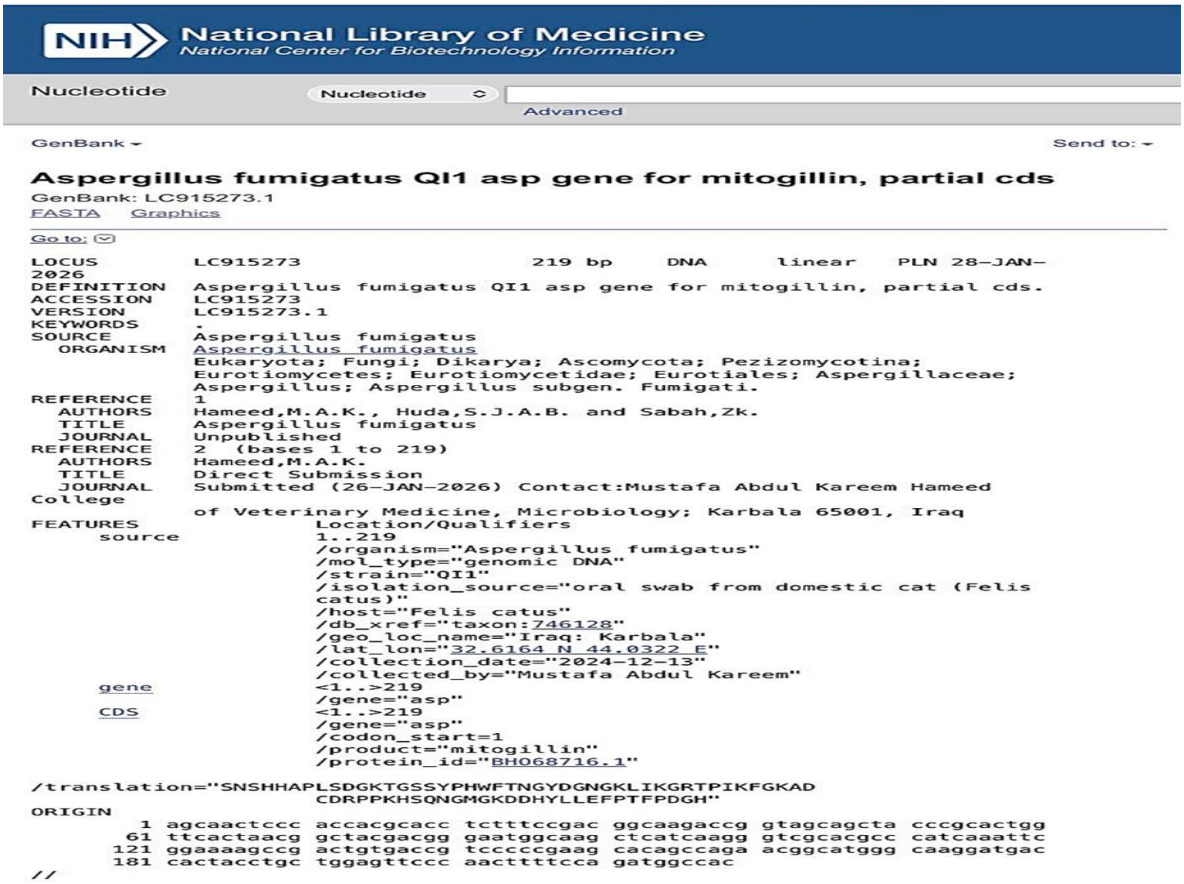
Appendix 2: Registration of alkaline protease (alp1 gene) of *A. fumigatus* within GenBank /NCBI



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Nucleotide | Nucleotide | Advanced

GenBank | Send to:

Aspergillus fumigatus QI1 asp gene for mitogillin, partial cds

GenBank: LC915273.1
FASTA Graphics

Go to:

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2026
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            Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae;
            Aspergillus; Aspergillus subgen. Fumigati.
REFERENCE   1
  AUTHORS   Hameed,M.A.K., Huda,S.J.A.B. and Sabah,Zk.
  TITLE     Aspergillus fumigatus
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 219)
  AUTHORS   Hameed,M.A.K.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JAN-2026) Contact:Mustafa Abdul Kareem Hameed
College
            of Veterinary Medicine, Microbiology; Karbala 65001, Iraq
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Appendix 3: Registration of Aspartic protease (asp gene) of *A. fumigatus* within GenBank /NCB