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Molecular Study of *F. gigantica* Parasite Isolated from Slaughtered Cows and Buffaloes by COX1 Gene and Phylogenetic Tree Analysis in Nasiriyah City / Iraq

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Abstract

Objectives

Fasciolosis is a parasitic disease caused by the liver flukes Fasciola hepatica and Fasciola gigantica. These parasites primarily affect ruminants such as cattle and sheep but can also infect humans, making it a notable zoonosis. The disease is typically transmitted through the ingestion of water or vegetation contaminated with the larval stages of the parasite. Fasciolosis poses substantial economic and health challenges, particularly in regions where livestock farming is prevalent.

Materials and methods

In a recent study conducted in Nasiriyah, Iraq, 30 samples of Fasciola gigantica were isolated from infected cows and buffaloes at the central abattoir. The primary aim was to identify and characterize the common strains of F. gigantica present in these animals using Polymerase Chain Reaction (PCR) technology. By focusing on gene sequences, particularly the Cytochrome C Oxidase Subunit 1 gene (COX1), researchers sought to compare these strains with those registered in the NCBI-BLAST database through phylogenetic tree analysis.

Results

The PCR technique successfully amplified DNA extracted from F. gigantica samples, enabling further genetic analysis. The study's results highlighted the genetic relationships between the Iraqi isolates and those from other regions. For instance, one isolate, identified as F. gigantica number 1 with the sequence number OQ152484, showed a 100% match with an Iranian isolate (OP903335) registered in the NCBI-BLAST GenBank. Another isolate, numbered OQ152485, was identical to a Turkish strain. Additionally, isolates 3 and 4 (OQ152486 and OQ152487) were closely related to Iranian isolates OQ070200 and OP903336, showing a similarity of 99.41% and 99.71%. Lastly, isolate number 5 (OQ152488) was found to be 99.41% identical to an Indian isolate (KU363230) recorded in the database.

Conclusions

These findings underscore the genetic diversity and potential geographic distribution of F. gigantica strains, emphasizing the importance of continued surveillance and genetic characterization to better understand the epidemiology of fasciolosis and inform control strategies.

Keywords: Buffaloes, Cows, Genetic diversity, PCR Technology, Zoonosis

Paper Type: Research Paper.

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Introduction

F. gigantica is characterized by its leafy shape as it invades the liver and then heads to the bile ducts to settle and complete its maturation there (Olaogun et al. 2022). It is larger than the *F. hepatica* and can reach 7.5 cm in length and its eggs are large and have a size of 190×100 μ, while *F. hepatica* is 1-2 mm long when it enters the liver. Despite the similarity of the two species of

the genus *Fasciola* spp. in morphologic and anatomic characteristics, there are several differences between them, including that *F. gigantica* has a more transparent body and indistinct shoulders, and the oral volume is smaller than its counterpart in *F. hepatica*, and the ventral sucker is larger (Over et al. 1982). The body is more pointed and the location of the testicles is more advanced. During its life cycle, the *F. gigantica* parasite needs two hosts: an intermediate host is the most important aquatic snail *Lymnaea auricularia* (Imani-Baran et al. 2011) and a definitive host of mammals such as humans, cattle, goats, sheep and buffaloes. The importance of different intermediate hosts depends on their different geographic distribution due to climate (tropical or temperate) (Schweizer et al. 2005), temperature and type of habitat (deep, permanent bodies of water versus swampy areas that sometimes dry up) (Schweizer et al. 2005). It was recently reported Dermauw et al. (2021) that human fasciolosis is still a neglected disease and there is an urgent need to develop more epidemiological studies. However, studies on animal fasciolosis were limited to estimating the prevalence of the disease in live or slaughtered animals (De Matos et al. 2020). The symptoms are usually fever, loss of appetite, severe abdominal pain, weight loss and an enlarged liver. The life cycle includes the emergence of eggs without embryos with feces, then they grow in the water and hatch into pear-shaped larvae called Miracidia, then they search for the middle host, the snail *L. auricularia,* after which they pass through several stages, namely the Rediae stage and the second generation of Rediae, the stage of Cercaria and Metacercaria. Cercaria released from the snail into the water and loses its tail to sac on plants or sac directly in the water and remains there until ingestion by the definitive host, Metacercaria become infective 24 hours after they become cystic. Describe the molecular structure of F. hepatica and F. gigantica based on the COX1 gene previously in Iraq (Mohammed et al. 2016; Muhammad and Hassan 2021), Egypt (Dar Y et al. 2012) and Saudi Arabia (Shalaby and Gherbawy 2013).

Some studies took a much broader view, applying molecular systematics to all flatworms (Platyhelminthes), including Monogenea, Digenea, and Cestoda (Nolan and Cribb 2005). Moreover, the epigenome comprising different mechanisms e.g. DNA methylation, remodeling, histone tail modifications, chromatin microRNAs and long non-coding RNAs, interact with environ-mental factors like nutrition, pathogens, climate to influence the expression profile of genes and the emergence of specific phenotypes (Barazandeh et al. 2016a; Mohammadinejad et al. 2022; Akin et al. 2020). Multi-level interactions between the genome, epigenome and environmental factors might occur (Mohamadipoor et al. 2021; Mohammadabadi & Tohidinejad et al. 2017; Jafari Ahmadabadi et al. 2023). Furthermore, numerous lines of evidence suggest the influence of epigenome variation on health and production (Barazandeh et al. 2016b; Safaei et al. 2022; Bordbar et al. 2022). Also, the information obtained from the analysis of biological data by

bioinformatics, in aligning sequences in information banks to find genetic similarities and differences, predicting the structure and function of gene products (Mohammadabadi et al. 2021; Shokri et al. 2023; Mohammadabadi et al. 2024) and it helps to find the phylogenetic relationship between genes, transcriptome and protein sequences (Mohammadabadi et al. 2023; Amiri Roudbar et al. 2020). Serology was the only diagnostic tool used in many human case reports (Kabaalioglu et al. 1999), but we were unable to do serologic testing for fascioliasis on any of our patients since the test was unavailable in our province when the patients presented. One further tool in the diagnostic toolbox is the 99mTc uptake liver scan, which may reveal regions of abnormal radio colloidand/or lacunar structure. Diseased liver characteristics, such as surface tunnel-like lesions and, more specifically, yellow-white nodules with a halo hyper-vascularized of varying sizes and shapes, may be seen by laparoscopy. Liver, peritoneum, and other Glisson capsule lesions may all be biopsied simultaneously for diagnosis (Cosme et al. 2001). Fascioliasis, according to histological examination, differs from other well-known illnesses such as TB, toxoplasmosis, sarcoidosis, and non-Hodgkin's lymphoma by the presence of granulomas. Common histological findings include eosinophil and inflammatory infiltration around Charcot-Leyden crystals, cellular debris, and central necrosis.

In 1964, the first case of fascioliasis was documented in Iraq. An immature Fasciola gigantica worm caused an ectopic case of human fascioliasis in the eye (Fattah et al. 1964). The first instance of biliary fascioliasis was described in 2004 (Hawrami et al. 2004). It was unexpectedly identified during investigation. When Ezzat et al. initially detailed endoscopic ERCP care, it was a huge step forward (Ezzat et al. 2010). It was reported in 2010 that during a standard cholecystectomy, an adult Fasciola hepatica worm was discovered within the gallbladder (Hawrami et al. 2010). The aim of the study is to study multiple sequence alignments of the COX1 gene to show the presence of nucleotide base sequence variations between the genotypes of *F. gigantica* parasite isolated from buffaloes and cows.

Materials and methods

Collection of samples from slaughtered cows and buffaloes: Thirty samples of *F. gigantica* were collected from buffaloes and cows infected animals from Al-Nasiriyah Central Slaughterhouse (Figure 1). These samples were transported by refrigerated plastic cases or directly from the slaughterhouse to the laboratory. A PCR technique was performed to detect the genotypes of *F. gigantica* based on the COX1 gene.

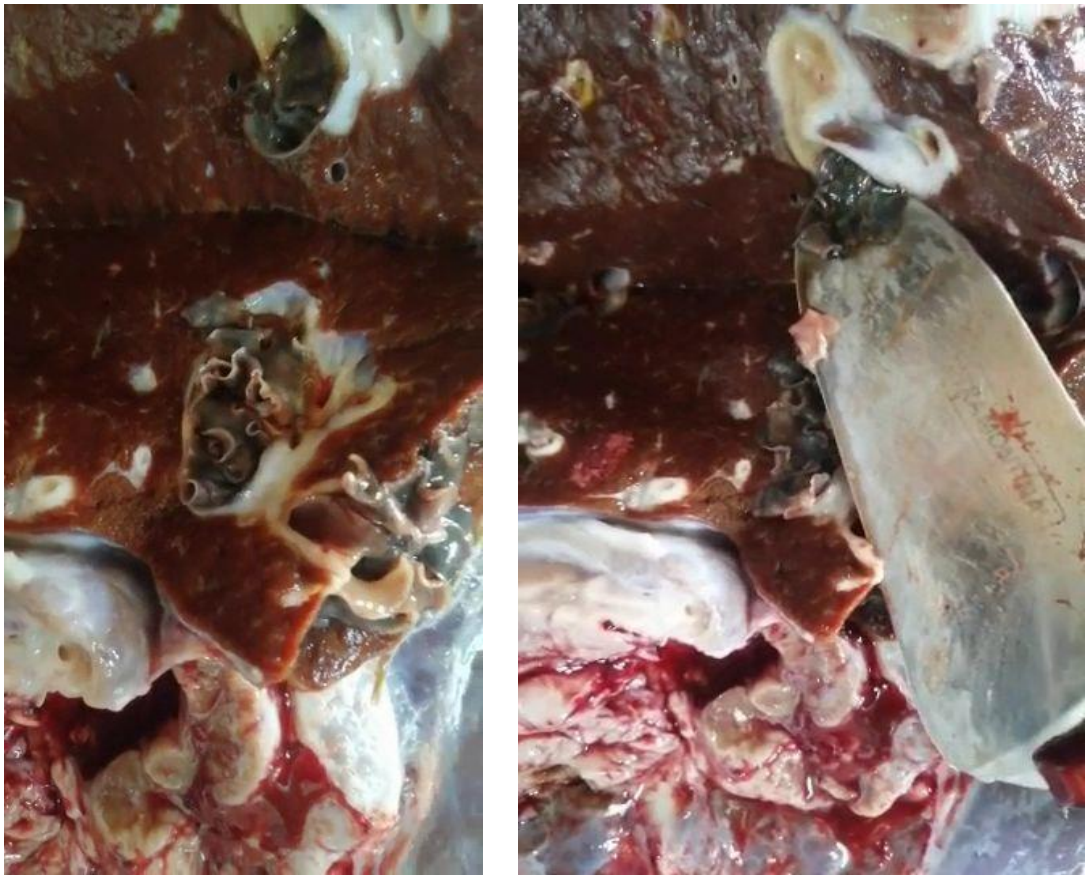

Figure 1. Samples of *F. gigantica* in infected buffaloes and cows

Isolation of Fasciola gigantica parasite-Primers: The specified primers listed in Table 1 were designed based on Primer3Plus and were used to determine the genus F. gigantica and its genotypes (Simsek et al. 2011).

Table 1. Molecular determination gene primers for PCR assay

Primer name / *F. gigantica*	Sequence 5-----3	Target gene	Product Size	Accession Number
Forward	TTTTTTGGGCATCCTGAGGTTTAT	COX1	947bp	KX021280-
Reverse	TAAAGAAAGAACATAATGAAAATG	490		KX021299

PCR components: The polymerase chain reaction (PCR) was performed in 20 µL reaction mixture containing 1 µL each of each primer (0.5 pmol/20 µL), 10 µL master mix (Macrogen in Korea), and 50 ng gDNA. The amplification conditions included initial denaturation at 95 °C for 5 minutes then followed by 38 cycles each of denaturation at 95 °C for 35 seconds, annealing at

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53 °C for 35 seconds, extension at 72 °C for 35 seconds and then final extension at 72 °C for 5 minutes.

DNA extraction and amplification of COX1 gene: Each worm was placed in individual eppendorf tubes, lysed with a lysis solution plus proteinase K, and then incubated at 56 °C for 3 hours until tissue lysis was complete. Then, 200 µL of binding solution was added to the clear lysates and mixed thoroughly with a Vortex mixer for 15 seconds. Then it was incubated at 56 °C for 10 minutes. 200 µL of absolute ethanol was added to each tube and mixed thoroughly. The centrifuge was used at 13,000 rpm for 1 minute. Finally, the DNA was precipitated by centrifugation at 13,000 rpm for 1 min and kept at -20 °C for further analysis (Hasanpour et al. 2020).

Amplification of DNA by PCR Thermocycler: In this study, the DNA amplification process involved the use of a PCR thermocycler, employing specific temperature cycles to replicate the COX1 gene from samples of Fasciola gigantica. The process commenced with an initial denaturation stage at 95°C to separate DNA strands, followed by iterative cycles of denaturation, annealing at 53°C for primer binding to the DNA template, and extension at 72°C to synthesize new DNA strands, as outlined in Table 2 provided. A final extension phase ensured thorough synthesis. These exact thermal conditions were pivotal in achieving successful and efficient amplification of the target gene.

Table 2. PCR thermocycler conditions

PCR step	Tem.	Time	Repeat
Initial denaturation	95ºC	5 min	1
Denaturation	95ºC	35 sec	
Annealing	53	35 sec	38
Extension	72ºC	35 sec	
Final extension	72ºC	5 min	1

Gel electrophoresis: The PCR product was analyzed by agarose gel by dissolving 1.5% agarose powder in 1x TBE buffer (100 mL) and microwaved to 95 °C for 2 minutes, after which it was allowed to cool and solidify. Then 5 µL of PCR products were added into each comb. Finally, the PCR products were visualized by the gel documentation system.

Isolation of Fasciola gigantica parasite-Amplicon sequencing and phylogeny analysis: Five positive PCR samples selected from the positive PCR samples for DNA were sent to

Macrogen in Korea in an ice bag by DHL for DNA sequencing by sanger sequencing system. Once the sequences were obtained, they were then sent to NCBI-Gen Bank to obtain Genbank accession numbers and compare them with other different world strains. DNA sequence analysis (phylogenetic tree analysis) was performed using Molecular Evolutionary Genetics Analysis version 10 (megax) and multiple sequence alignment analysis based on Clustal W alignment analysis. The taxonomy of specific species was analyzed by phylogenetic tree analysis in comparison with known NCBI-Blast sequences (Stecher et al. 2020).

Results and discussion

Result of PCR of COX1 gene: The results of the polymerase chain reaction (PCR) technique, after electrophoresis was performed on an agarose gel (1.5% agarose), successfully amplified the DNA extracted from *F. gigantica* samples for the COX1 gene at a size of 490 base pairs. As the results were positive in all samples (Figure 2).

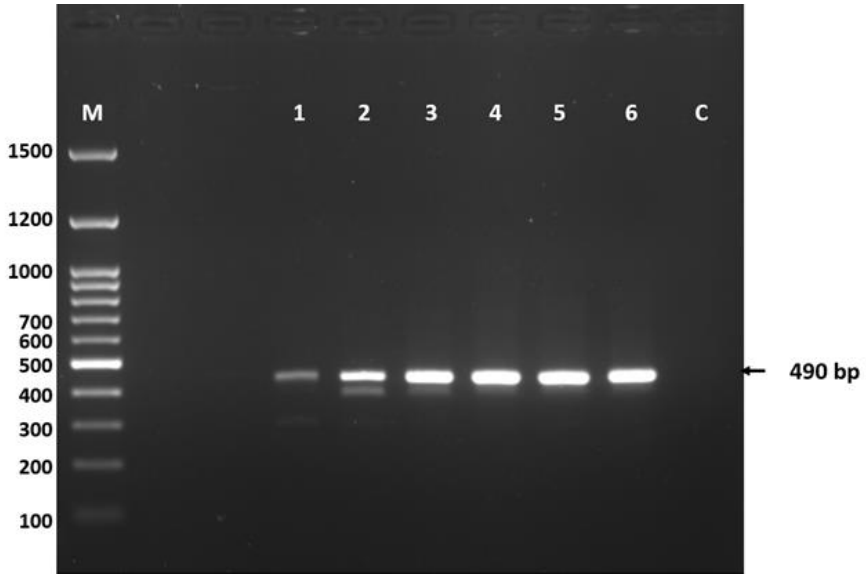

Figure 2. Gel electrophoresis image (1.5 % agarose) shows the amplicon of positive samples of *F. gigantica* targeting the COX1 gene in size 490 bp

DNA sequencer technique & Phylogenetic tree analysis: Only 5 positive samples were sent to the Korean company Macrogen for DNA sequencing. Then, those samples were registered in the NCBI-BLAST Genome Bank for genetic tree analysis, and they were registered under the sequence numbers OQ152484, OQ152485, OQ152486, OQ152487, OQ152488. *F. gigantica* isolate number 1 with sequence number OQ152484 in the NCBI-Blast showed a 100% match with the Iranian isolate registered in the NCBI-Blast International Gen Bank with sequence number OP903335. Isolate number 2 with sequence number OQ152485 is identical to the Turkish

isolate. The isolates (3 and 4) with sequence numbers OQ152486 and OQ152487, respectively, are identical to the Iranian isolates numbered OQ070200 and OP903336 in the NCBI-Blast, with a matching percentage of 99.41 and 99.71%, respectively. 5 with serial number OQ152488 is identical to the Indian isolate recorded in NCBI-Blast and bearing serial number KU363230 with a match rate of 99.41. As in Table 3.

Table 3. The NCBI-BLAST Homology Sequence identity (%) between local *F. gigantica* sequences were deposited in gene bank

		NCBI-BLAST Homology Sequence identity (%)			
Sequence name	**Accession number**	**Identical to**	**Genbank Accession number**	**Country**	**Identity (%)**
1	OQ152484	*Fasciola gigantica*	OP903335	Iran	100
2	OQ152485	*Fasciola gigantica*	GQ121277	Turkey	99.71
3	OQ152486	*Fasciola gigantica*	OQ070200	Iran	99.41
4	OQ152487	*Fasciola gigantica*	OP903336	Iran	99.71
5	OQ152488	*Fasciola gigantica*	KU363230	India	99.41

The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al. 2004; Tamura et al. 2021; Margiana et al. 2022; Arif et al. 2023; Lei et al. 2022; Bashar et al.2022; Lafta et al. 2023; Abbas et al. 2022; Hussein et al. 2022). This analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 339 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Hjazi et al. 2023; Al Anazi et al. 2023; Althomali et al. 2023; Hjazi et al. 2023; Gupta et al. 2023; Sane et al. 2023; Al-Jassani et al. 2022; Ze et al. 2023; Al-Dolaimy et al. 2024; Al-Hawary et al. 2023; Zaman et al. 2023; Muzammil et al. 2023). Phylogenetic tree analysis of *F. gigantica* based on the (COX1) gene for the currently identified sequences indicated in Figures 2 and 3 as red circles. These have been deposited in the NCBI-BLAST International Gen Bank as can be seen as sequence numbers. These were compared with previously deposited single sequences from Iran and Turkey (blue circles), analyzed by Mega X using the Bootstrap method. At a total genetic heterogeneity of 0.00050.

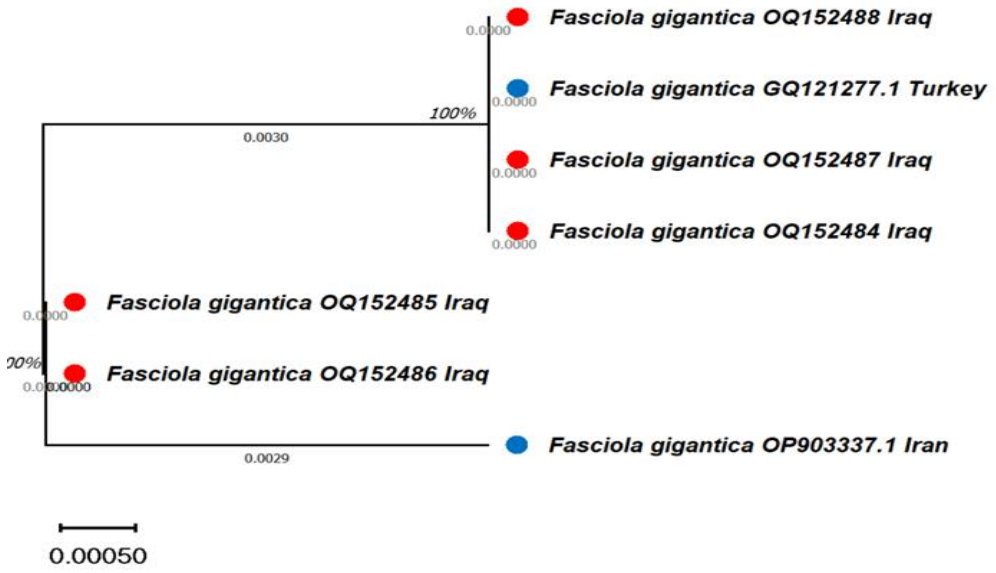

Figure 3. Phylogenetic tree analysis of F. gigantica based on the (COX1) gene for the currently identified sequences

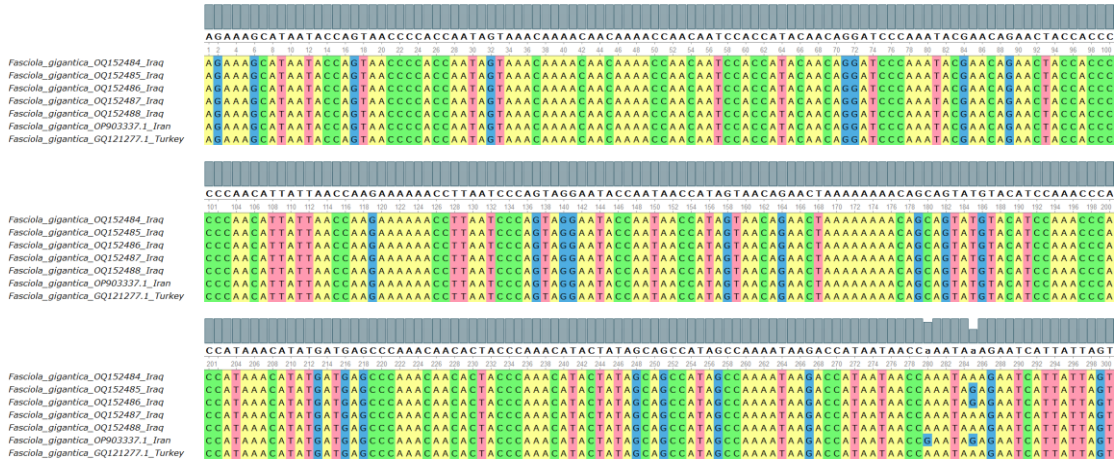

Figure 4. Multiple sequence alignment of the identified *F. gigantica* in comparison with homologues global sequence from Iran and Turkey. Highlighting with four colours to indicate the similarity. This was analysed by Mega X

The 30 samples that were submitted to the NCBI BLAST GenBank database with the accession numbers OQ152484 to OQ152488 were confirmed to be Fasciola hepatica. For example, an isolate from Iran (OP903335) that is part of the NCBI-BLAST GenBank had a perfect match with another isolate from the same species (F. gigantica number 1), which was recognized with the sequence number OQ152484. Another sample, with the ID number OQ152485, was a replicate of a strain found in Turkey. Isolates 3 and 4 (OQ152486 and

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OQ152487) also exhibited a high degree of similarity (99.41% for OQ070200 and 99.71% for OP903336) with their Iranian counterparts. In conclusion, the fifth isolate (OQ152488) was determined to be 99.41% similar to an isolate (KU363230) from India that was already included in the database.

Conclusion: These findings underscore the genetic diversity and potential geographic distribution of F. *gigantica* strains, emphasizing the importance of continued surveillance and genetic characterization to better understand the epidemiology of fasciolosis and inform control strategies.

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Conflict of Interest: There is no conflict of interest.

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مطالعه مولکولی انگل F. gigantica جدا شده از گاوها و گاومیش های ذبح شده توسط ژن COX1 و آنالیز درخت فیلوژنتیک در شهر ناصریه / عراق

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چکیده

هدف: فاسیولوزیس یک بیماری انگلی است که توسط فلوک های کبدی Fasciola hepatica و Fasciola gigantica ایجاد می‌شود. این انگل‌ها در درجه اول بر نشخوارکنندگان مانند گاو و گوسفند تأثیر می گذارند، اما می‌توانند انسان را نیز آلوده کنند و آن را به یک بیماری مشترک بین انسان و دام تبدیل کنند. این بیماری معمولاً از طریق بلع آب یا پوشش گیاهی آلوده به مراحل لاروی انگل منتقل می‌شود. فاسیولوزیس چالش‌های اقتصادی و بهداشتی قابل توجهی را ایجاد می‌کند، به‌ویژه در مناطقی که دامداری رایج است.

مواد و روش‌ها: در مطالعه‌ای که اخیراً در ناصریه عراق انجام شد، ۳۰ نمونه Fasciola gigantica از گاوها و گاومیش‌های آلوده در کشتارگاه مرکزی جدا شد. هدف اولیه شناسایی و مشخص کردن سویه‌های رایج F. gigantica موجود در این حیوانات با استفاده از فناوری واکنش زنجیره‌ای پلیمراز (PCR) بود. محققان با تمرکز بر توالی‌های ژن، به ویژه ژن سیتوکروم C اکسیداز زیرواحد ۱(COX1) ، به دنبال مقایسه این سویه‌ها با آنهایی بودند که در پایگاه داده NCBI-BLAST از طریق تجزیه و تحلیل درخت فیلوژنتیکی ثبت شده‌اند.

نتایج: DNA استخراج شده ز نمونه‌های F. gigantica با تکنیک PCR با موفقیت تکثیر شد و امکان تجزیه و تحلیل ژنتیکی بیشتر را فراهم کرد. نتایج این مطالعه روابط ژنتیکی بین جدایه‌های عراقی و سایر مناطق را برجسته کرد. به عنوان مثال، یکی از جدایه‌ها، با نام F. gigantica شماره ۱ با شماره توالی OQ152484، تطابق ۱۰۰٪ با ایزوله ایرانی (OP903335) ثبت شده در بانک ژن NCBI-BLAST را نشان داد. ایزوله دیگر با شماره OQ152485 مشابه سویه ترکیه‌ای بود. علاوه بر این، جدایه‌های ۳ و ۴ (OQ152486 و OQ152487) با جدایه های ایرانی OQ070200 و OP903336 ارتباط نزدیکی داشتند که شباهت ۹۹.۴۱٪ و ۹۹.۷۱٪ را نشان دادند. در نهایت، ایزوله شماره ۵ (OQ152488) ۹۹/۴۱ درصد با یک ایزوله هندی (KU363230) ثبت شده در پایگاه داده یکسان بود.

نتیجه گیری: این یافته‌ها بر تنوع ژنتیکی و توزیع جغرافیایی بالقوه سویه‌های F. gigantica تاکید می‌کند و بر اهمیت نظارت مستمر و تعیین خصوصیات ژنتیکی برای درک بهتر اپیدمیولوژی فاسیولوزیس و اطلاع‌رسانی استراتژی‌های کنترل تاکید می‌کند.

واژه‌های کلیدی: بوفالو، گاو، تنوع ژنتیکی، فناوری PCR، زئونوز

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