https://doi.org/10.46389/rjd-2024-0012
www.jrdiabet.ro
Rom J Diabetes Nutr Metab Dis
2024; Volume 31, Issue 1, pages 12-20

MOLECULAR IDENTIFICATION OF FASCIOLA SPP IN CATTLE IN AL-DIWANYIA, IRAQ

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Abstract
The reading focused on taking isolates of cow liver from the butchers' market in the city of Diwaniyah for the period from Agest 2022 to April 2023 in order to diagnose flatworm infection, specifically genus *Fasciola*, which relied on molecular diagnosis as an polymerase chain reaction (PCR) for the specific identification of the parasite and based on the 16s RNA genetic region as a conservative region with a size of (433 bp). According to previous studies, which came after electrophoresis and showing the phylogenetic relationship with isolates recorded from Egypt, China, and America on the NCBI website, the results showed a high percentage of identity with the *Fasciola gigntica* species and similarity reaching from (98.7-99.16%).
Keywords: *Fasciola gegntica*,Molecular, flatworm,phylogenetic, polymerase

1. Introduction

F. hepatica and *F. gigantic* are frequent liver fluck and source of the parasitic disease fasciolosis ,which affects both human and animal (1). Fasciolosis's clinical characteristics might vary (acute, sub-acute, and chronic)(2). Sudden death may result from acute fasciolosis(3). Subacute is caused by eating a little amount of metacercaria and is characterized by anemia, jaundice, and malaise (4). It completes its life cycle in two different hosts ,a definitive host(Cattle, sheep, numerous other ruminants, equidae, swine, and rabbits),and an intermediate host (genus Lymnaea),and spreads to animals through food and water, where they are subsequently exposed to the infectious stage (4). Clinical signs, grazing history, seasonality, laboratory testing of feces, and postmortem examination are used to identify fascioliosis (5) . Fasciolosis pathogenesis varies with the parenchymal and biliary stages of parasite growth. The parenchymal phase, which is associated with liver injury and hemorrhage, happens when flukes migrate through the liver parenchyma. Because to the adult flukes' haematophagic behavior and the cuticular spines' injury to the bile duct mucosa, the biliary phase occurs simultaneously with parasite residence in the bile ducts (6). Fasciolosis is diagnosed based on clinical symptoms, grazing history, seasonal occurrence, laboratory testing of feces, and postmortem examination (5). We compare the evolutionary structure of the fasciola in Iraqi cattle to that of other locations.

2.Material and methods

Genomic DNA was extracted from 18 fasciola samples isolated from infected cattle . DNA extracted by means DNA extraction kit (tissue protocol) addBio, korea and done rendering to corporation instruction . PCR to identify fasciola .spp was done with large subunit RNA 16s gene (16s mitochondrial). Genomic DNA from liver fluke worm samples were extracted with DNA

extraction kite (AddBio, korea)preformed to instructions primers for PCR of fasciola .spp. larg subunit RNA 16s (16s mitochondrial) were taken from NCBI-Gen bank(MN970008,AJ243016) and primer design online program . primers were provided by korea listed in Table 1 , PCR .master mix (AddBio ,korea)components are shown in Table 2

Table (1). Primers used for PCR

Primer name	Sequence 5-----3	Target gene	Reference in NCBI with accession number	
Forward	ATCATTACCTGAAAATCTACTCTCACA	large subunit RNA (16S mitochondrial)	*F hepatica*	*F gigantica*
Reverse	GTACGTATGGTCAAAGACCAGGTT	433 bp	AJ243016.1	MN970008

Table(2). Components of PCR Reaction .

PCR component	Volume/ µl
Master mix	10
Forward primer (0.5 pmol/20 µl)	2
Reverse primer (0.5 pmol/20 µl)	2
PCR water	4
DNA template (100 ng)	2
Total	20

PCR master mix was equipped with Taq DNA polymerase , dNTPs , Tris-HCI PH:9,0 , KCO , MgCl2, stabilizer , and tracking dye PCR tubes then vortexed (Exispin vortex centrifuge, CYAN, Belgium) at 3000 rpm for 3 minutes and before thermocycler conditions with conventional PCR Thermocycler are listed in the table 3 below

Table (3). **PCR thermocycler conditions.**

PCR step	**Temperature**	**Time**	**Repeat**
Initial denaturation	95°C	2 min	1
Denaturation	95°C	30 second	38
Annealing	55	30 second	

Extension	72°C	30 second	
Final extension	72°C	5min	1

3.Results

A-Gel electrophoresis technique

Based on molecular techniques and the accuracy of the diagnosis of the parasite, the size of 433 bp for the region of the large subunit RNA 16S gene (1 – 8) samples within the gel electrophoresis, as shown in Figure (1)

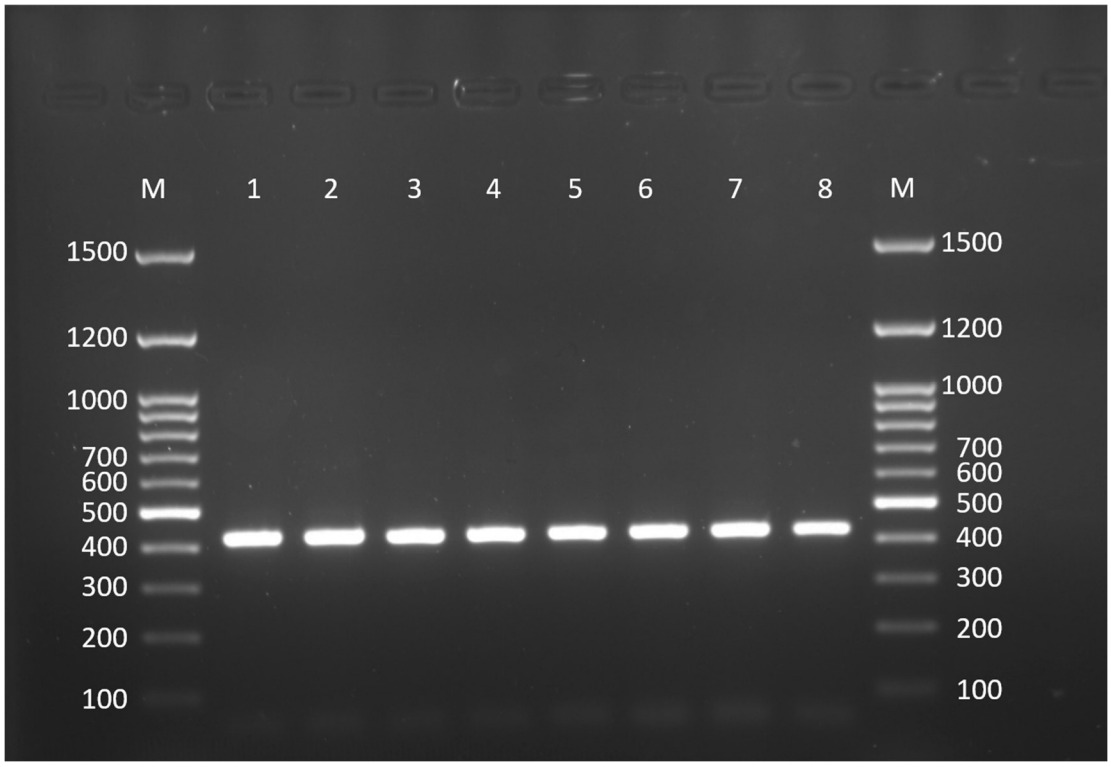
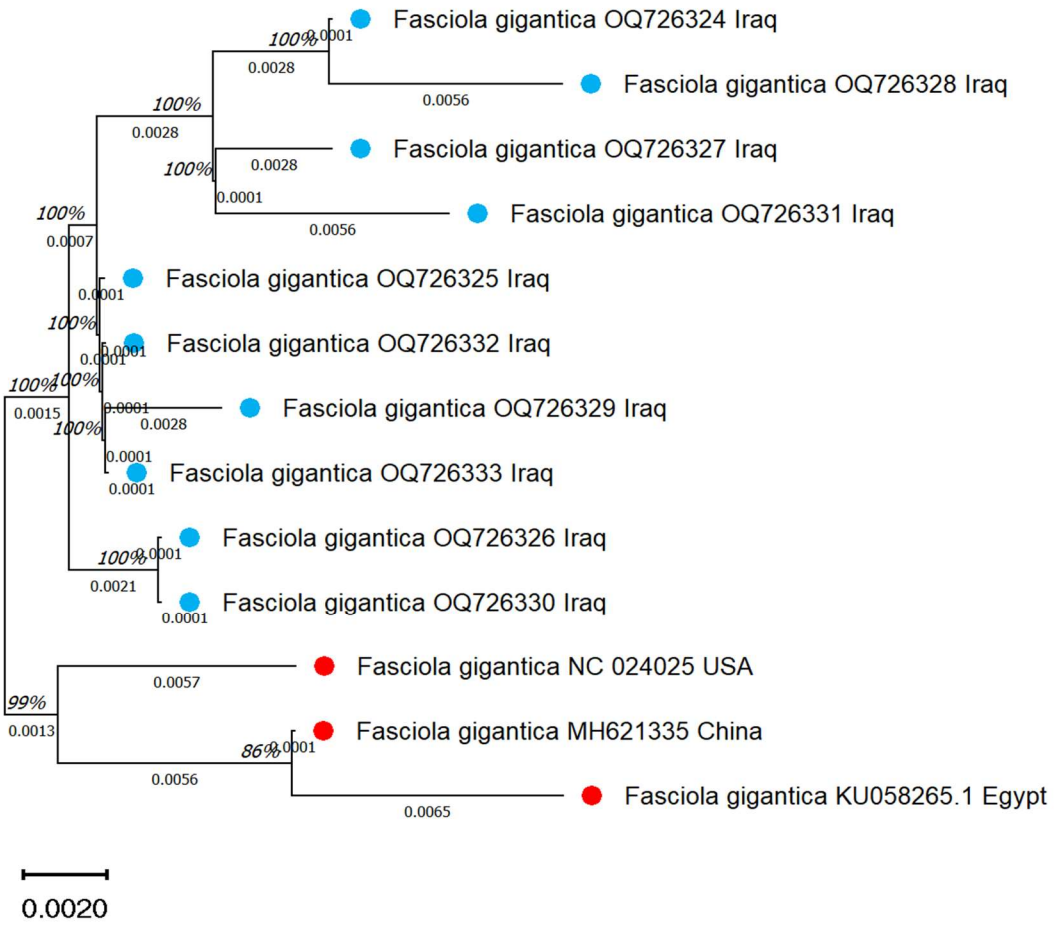


Figure 1. : Gel electrophoresis image (1.7 % agarose) shows the amplicon of some positive samples (lanes= 1-8) of *Fasciola sp.* targeting the large subunit RNA 16S gene in size 433 bp.

B- Phylogenetic tree analysis and BLAST identity

Through the design of a phylogenetic tree, the study showed the overlap between the global isolates documented on NCBI and the local isolates include (OQ726324, OQ726325,OQ726326, OQ726327, OQ726328, OQ726329, OQ726330, OQ726331, OQ726332,OQ726333) and at the same distance from an ancestor with its two branches, as indicated in figure (5). By using NCBI-BLAST software for the purpose of matching sequences, the study presented that the percentage ranged between 99.16 - 98.7 with isolates from different areas around the world, including the

USA, China, Egypt, and Japan, as indicated in Table (4).



Figure(2) : phylogenetic tree analysis of *Fasciola gigantica* targeting the (Large subunit RNA 16S) gene of the currently known sequences mentioned as blue circles. These were put in the global gene bank as can be understood as accession numbers. These were likened with the only before deposited sequence from Egypt , China , and USA (red circles). These were analysed by Mega X using Bootstrap method.

Table (1): the NCBI-BLAST Homology Sequence identity (%) between local *Fasciola gigantica* sequences were deposited in gene bank under accession numbers (OQ726324, OQ726325, OQ726326, OQ726327, OQ726328, OQ726329, OQ726330, OQ726331, OQ726332, OQ726333) and NCBI-BLAST only deposited sequence from (Egypt, China, and USA).

Sequence name	Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Gen bank Accession number	Country	Identity (%)

1	OQ726324	*Fasciola gigantica*	KU058265.1	Egypt	98.7
2	OQ726325	*Fasciola gigantica*	NC_024025	USA	99.16
3	OQ726326	*Fasciola gigantica*	MH621335	China	99.16
4	OQ726327	*Fasciola gigantica*	KU058265.1	Egypt	98.7
5	OQ726328	*Fasciola gigantica*	KF543342	China	98.32
6	OQ726329	*Fasciola gigantica*	NC_024025	USA	98.88
7	OQ726330	*Fasciola gigantica*	NC_024025	USA	99.16
8	OQ726331	*Fasciola gigantica*	MH621335	China	98.60
9	OQ726332	*Fasciola gigantica*	KF543342	China	99.16
10	OQ726333	*Fasciola gigantica*	LC649568	Japan	98.32

Figure 3: Numerous sequence alignment of the recognised *Fasciola gigantica* contrast with homologues global sequence from Egypt, China , and USA. Highlighting with

four colours to show the resemblance. This was studied by Mega X. show in sequence with accession number OQ726331 there is mutation in site 107 in which T mutated in to A, In sequence with accession number OQ726324, and OQ726328 there are mutation in site 118 in which G mutated in to T, In sequence with accession number OQ726331 there is mutation in site 135in which T mutated in to G, and In sequence with accession number OQ726329 there is mutation in the site 164 in which C mutated in to T

4. Discussion

Because of the increased infection risks, animal fascioliasis, a neglected international illness, has become an important food safety challenge for zoonotic transmission and many susceptible hosts. F. hepatica and F. gigantica are the two species most frequently cited as the etiological agents of fasciolosis(1). The biology and the complex evolutionary cycle of this parasite encouraged researchers to study the estimated stability or genetic variation that may appear within the population groups of these flukes, and factors that limit them: First, F. hepatica has the potential for cross- and auto-fertilization in addition to its usual spermatogenetic capacities. It is capable of parthenogenesis under specific conditions, and this mode of reproduction could influence the DNA frequencies in some populations (7,8,9). Additionally, F. hepatica's requirement to consume intermediate hosts (snails) and final hosts to complete its life cycle may encourage higher variation. Furthermore, Fasciola spp. adult worms may remain in hosts for a long period of time, and untreated animals can produce vast quantities of eggs in their feces, which could lead to genotypic variety within the worms. Variations in the environment, including those related to altitude temperature, humidity, and farming practices, may potentially affect the population framework of flukes (10,11,12). Phylogenetic tree analysis of DNA isolates seen in Figure (2), Large subunit RNA 16S gene sequences prepared of 10 isolates with the identity of FASCIOLA SPP which were deposited in gene bank under accession numbers (OQ726324 - OQ726333) from cattle. The current findings of the molecular methods have confirmed infection of *Fasciola gigantica*, PCR amplicon of some positive samples (lanes= 1-8) valued the large subunit being 433bp in DNA size, that very linked to them of the same clade (monophyly) of the phylogenetic tree, in contrast, a genetic relationship originated to a second clade included USA, china, and Egypt, depending on the Large subunit RNA 16S gene isolates of cattle liver in diwaniyah /Iraq. In the table (1), alignment of the different isolates was reported (98.7-99.16%) comparable with nucleotide sequences (accession number: Egypt KU058265.1, USA NC_024025, china MH621335, KF543342, japan LC649568). The study did not identify a clear discrepancy between the local isolates, confirming the existence of a transient epidemiological case from other areas of infection and high population genetic retention of Fasciola adults, and the approximate distance from the ancestors of some isolates compared to samples from other areas.

Some taxon indicate a state of increasing mutations that prepare for possible future variation, confirming what we have shown from the previous factors. As a result, scientists considered that F. gigantica was presently common in the Middle East, Asia, and Africa (13). our results are in a good agreement with many authors as (14) when analyzed various regions of the 18S rRNA gene

found that only 0.3% difference of local Iranian samples when compared with global isolates. Also, Elkhtam and Khalafalla in a genotyping study of goat isolates of *F. gigantica* in Egypt by utilizing an 18S rRNA gene with pairwise and multiple alignments reveals high sequence homology, up to 99.9% matched to the international isolates and *F. gigantica* (15) and study of infection from cattle in Aqrah city (16). but relatively dissimilar to (17,18).

5.Conclusion:

1. PCR method is significant approach in genomic variation detection of fasciola spp and using large subunite RNA 16s gene (16s mitochondrial) for fasciola giagintica of differentiating between fasciola spp
2. the sequenced regions were deposited in national center for Biotechnology information (NCBI) for the first time in Iraq under successive accession number form OQ726324 to OQ726333
3. the phylogenetic anylasis revealed new intermediate from of fasciola giagintica strain for the first time in Iraq and identified ahigh genentica similarity between Iraq strain and Egypte ,Chain ,and USA strain

Novelty

This research is new, original in detection *Fasciola gegntica* in cattle in AL-Diwanyia, Iraq.

Authors Contribution

All authors contributed equally to the manuscript and agree to the conditions outlined in the copyright assignment form included.

Conflict of interest

The authors have declared no conflict of interest.

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