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Sequencing strain *Fasciola* species in Cattle in Iraq

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Author

Ismael WaadUllah Ismael

Department of Anesthesia Techniques, College of Health and Medical Techniques/ Baghdad, Middle Technical University, Baghdad, Iraq.
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Corresponding Author Email Address: ismana.84@mtu.edu.iq

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Abstract

Fascioliasis is a significant zoonotic disease caused by Fasciola species that affects humans and animals like sheep, cows and buffaloes worldwide, causing public and veterinary health problems. The aim of this study is to focus on the genetic differentiation between the two species of Fasciola (F. hepatica and F. gigantica) by using the large subunit of 28S rDNA gene. A total of 45 adult worms were isolated from the liver of different cattle in Iraq-Basrah Province. Extraction of genomic DNA using a special kit was performed to isolate Fasciola species with 21 bp specific primers included that target 28S rDNA. The BLAST and PCR products showed evolutionary phylogenetic and genetic variation with similarity in about 80% between the two species of Fasciola. The results phylogenetically concluded a highly species-specific conservation and polymorphism with possible strain hybridization.

Keywords: *F. hepatica, F. gigantica*, Sequencing, Cattle.

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Introduction

Fasciola species is a trematode parasite that has a distinct morphological appearance. The disease caused by *Fasciola* is considered an important food and water parasitic zoonosis introduced via *Fasciola* genus, which belongs to the trematode liver flukes and has a wide distribution in tropical regions that have been recorded in several countries around the world (1).

According to the public health problem of the *Fasciola* diseases, the diagnosis of the *Fasciola* species (*F. gigantica* and *F. hepatica)* is considered a primary target for determining the phenotypic and genotypic variation and application of good preventive measures for control of the disease (2).

Because of the inability to differentiate between the two species by morphological examination, a wide range of genetic approaches were developed to demonstrate the degree of differences at the DNA and RNA molecular level by using the more advanced real-time PCR, which is considered a very accurate, easy, and slightly inexpensive methods to split between *F.hepatica* and *F.gigantica* dissimilarities (3).

The second internal transcribed spacer (ITS-2) of nuclear DNA is broadly used to differentiate between the two species of *Fasciola* reported as 1.7% sequence differences (4).

Fasciolosis, according to the World Health Organisation (WHO), is considered a first parasitic disease among many infections that are distributed epidemically in many areas around the world, like Africa, Asia and Europe (5).

Fasciola life cycle starts with lying eggs which is unembryonated and excreted in the faeces of final host to the external watery environment, then these eggs transferred to the embryonated stage, in addition to hatching of miracidia inside the snail of intermediate host, inside the snail the parasite develops asexual sporocysts and cercariae, finally cercariae leave the snail in the form of mature metacercariae as a final infective stage (6,7).

The complete life cycle of *Fasciola* involves two types of hosts: the final or definitive host represented by cattle, and an intermediate host, which is a freshwater snail. The infective metacercaria can transfer to and infect humans and animals by drinking polluted water or eating contaminated watery plants containing the metacercaria (8).

In the current study, genetic heterogeneity of both *Fasciola* species isolates can be established by using a small region of the manual 28S rDNA, so the ITS region between 5.8 S and 28.5 S of a nuclear ribosomal DNA was used as a conserved sequence to differentiate the polymorphism of *Fasciola hepatica* and *Fasciola gigantica* isolates in various ITS fragments (9-11).

Materials and Methods

Parasites :45 adult worms of liver flukes were isolated from the liver and gallbladder of both slaughtered cattle in Iraq, Basrah governorate. The study was approved by the Ethical Committee of the Middle Technical University/ College of Health and Medical Techniques, Baghdad.

DNA extraction : The Qiagen/ Germany mini kit of DNA extraction (QIAamp) was used in this study for extraction of genomic DNA from the isolated flukes according to the manufacturer's instructions of the company by amplification of small subunit Gene of 28s ribosomal DNA with a molecular weight of 610bp with as well as 21 bp forward and reverse primers, finally 2.5 µl from extracted DNA was purified via a nanodrop instrument for measurement of DNA concentration.

Primer design : Primers 20 bp of F (5`-ACGTGATTACCCGCTGAACT-3`) and 21 bp of R (5`-CTGAGAAAGTGCACTGACAAG-3`) were used (12).

PCR :The thermocycler PCR reactions for (5 samples) were conducted with 94 ° C for 3 min, and after that 30 cycles of 30 s at 94 °C, 30 s at 56.5 °C and 60 s at 72 °C. Finally, extension of primer for 5 mins was used. A Total of 12 microliters at 72 °C. PCR products were analyzed by electrophoresis at 1.5% conc. of agarose gel. A ladder with a molecular weight of 100 bp was included and viewed by ethidium bromide.

Results

In silico validation using NCBI Primer-BLAST confirmed the specificity of the primers, producing a single amplicon of 610 base pairs that fully matched the reference sequence of *Fasciola hepatica* isolate (GenBank accession: PV082153.1).This result supports that the sequenced PCR product corresponds to the correct target gene of the intended parasite. The forward primer aligned at position 1–20 and the reverse primer at position 610–590, covering the complete amplicon region as shown in Figure (1).

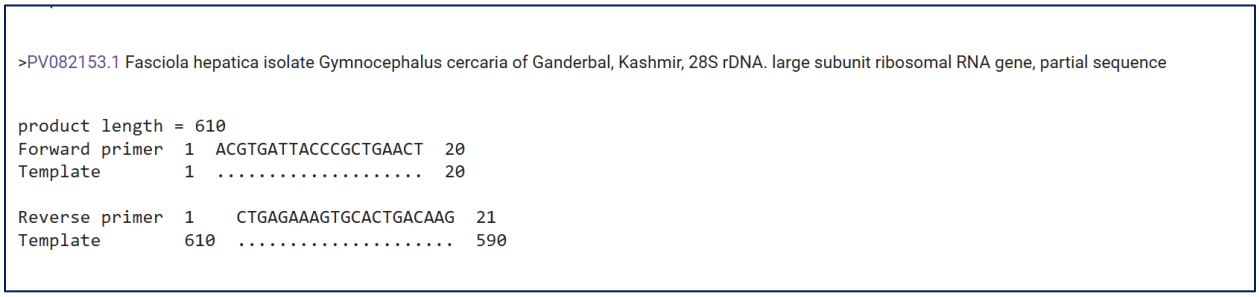

Figure 1. In silico validation of primer pair 28F and 28R on the 28S rDNA gene of *Fasciola hepatica* (GenBank: PV082153.1). The primers produced a specific 610 bp product matching the reference sequence, confirming that the correct target gene was amplified and sequenced.

Sample code 4

Fasciola sp. isolate S10 large subunit ribosomal RNA gene, partial sequence

Sequence ID: OR676767.1 Length: 597 Number of Matches: 1

Range 1: 33 to 471 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
388 bits(429)	1e-102	352/441(80%)	2/441(0%)	Plus/Plus

```
Query  25   AAGTAAAAAACCCGGAATTCCCTTATTAACGGCCAATGAAAGGGAAAAAACCCACCCCCC  84
Sbjct  33   AAGAAACTAACCAGGA-TTCCCTTAGTAACGGCGAGTGAACAGGGAAAAGCCCAGCACCG  91

Query  85   AAACCCGGGGTCCTTTGGCCCCTAAGCAATTTGGGGTTCAGGTTAACTCCCGAAAATTCT  144
Sbjct  92   AAACCAGTGGTCGTTTGGCCCCTAGGCAATGTGGTGTTCAGGTTAGCTCGCGAAGATGCT  151

Query  145  GCTCCCCCCTAAATCCTAAAATGGATAAGGTTACCCGGAAATGGCCCAATGAGGGTGAAA  204
Sbjct  152  GCTCCACCCTAAGTCCTATAATGAGTAAGGTTACTCGGACATGGCCCAATGAGGGTGAAA  211

Query  205  GGCCCCTGGGGGTGGAAATTTCAAATGGCCAATATTTTCCTGAACCAACCTTGGAATCCG  264
Sbjct  212  GGCCCGTGGGGGTGGAGATTCAGAATGGCCAGTATCTTCCTGAGCAGACCTTGGAGTCGG  271

Query  265  GttttttttGAATGCAGcccaaaacgggggggtaaactccctccaagggtaaaaactaacc  324
Sbjct  272  GTTGTTTGTGAATGCAGCCCAAAGCGGGTGGTAAACTCCATCCAAGGCTAAATACTAGCA  331

Query  325  ccaatcccaaaacaaaaaaatacccgggagggaaaatttaaaaaatacttttaaaaaaaaa  384
Sbjct  332  CGAGTCCGATAGCGAACAAGTACCGTGAGGG-AAAGTTGAAAAGTACTTTGAAGAGAGAG  390

Query  385  taaaaaaTGGGTGAAACCGTTTAAAAGTTAACAGGTGGAATTTAAATGGAAGCTCTAAAG  444
Sbjct  391  TAAACAGTGCGTGAAACCGTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTCTGAGG  450

Query  445  ATTTCCCTGGTAAATATGGCA  465
Sbjct  451  ATTCAGCTGGTGAGTATGGCA  471
```

Sample code 19

Fasciola hepatica isolate G15 28S ribosomal RNA gene, partial sequence

Sequence ID: HM369305.1 Length: 525 Number of Matches: 1

Range 1: 6 to 461 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
456 bits(505)	2e-123	374/457(82%)	3/457(0%)	Plus/Plus

```
Query  31   AACGAACCCGGATTCCCTTATTAACGGCAAATGAAAGGGAAAAAccccaccccccaaccc  90
Sbjct  6    AACTAACCAGGATTCCCTTAGTAACGGCGAGTGAACAGGGAAAAGCCCAGCACCGAAKCC  65

Query  91   cGGGGTCTTTTGGCCCCTAGGCAATGGGGGGTTCAGGTTACCTCCCGAAAATCCTGCTCC  150
Sbjct  66   TGTGGTCATTTGGCCCCTAGGCAATGTGGTGTTCAGGTTAGCTCGCGAAGATGCTGCTCC  125

Query  151  CCCCTAATTCCTTTAATGATTAAGGTTACCCGGAAATGGCCCAATGAGGGTGAAAGGCCC  210
Sbjct  126  ACCCTAAGTCCTATAATGAGTAAGGTTACTCGGACATGGCCCAATGAGGGTGAAAGGCCC  185

Query  211  CGGGGGGTGGAAATTCAAAATGGCCAttttttttCCTGAACGAACCTTGGAATCGGGtttt  270
Sbjct  186  GTGGGGGTGGAGATTCAGAAKGGCCAGTATCTTCCTGAGCARACCTTGGAGTCGGGTTGT  245

Query  271  ttttGAATGCACCCCAAACCGGGGGGTAAACTCCTTCCAAGGCTAAATACTAACCCaaat  330
Sbjct  246  TTGTGAATGCASCCCAAAGCGGGTGGTAAACTCCWTCCAAGGCTAAATACTAGCACRAGT  305

Query  331  ccaaaaccaaaaaattaccgggagggaaattttaaaattactttgaaaaaaaagtaaaaa  390
Sbjct  306  CCRATAGCGAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAARAGAGAGTAAACA  365

Query  391  TTTGTTGAAACCGTTTGAAGGTAAACAGTGTGG-GTTGATTTG-AAGTTTTGAGGATTTA  448
Sbjct  366  GTGCGTGAAACCGTTCARAGGTAAACAG-GTGGAGTTGAACTGCAAGCTCTGAGGATTCA  424

Query  449  CTTGGTGAGTATGGCATAAACTTGGTCATATTGGTTG  485
Sbjct  425  SCTGGTGAGTATGGCATGAGCTTGGTCATATTGGTTG  461
```

Sample code 45: No significant similarity found.

Sample code 50

Fasciola gigantica gene for 28S rRNA, partial sequence, isolate: KhuzBufa2G

Sequence ID: AB674553.1 Length: 580 Number of Matches: 1

Range 1: 11 to 460 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
453 bits(502)	8e-123	372/450(83%)	2/450(0%)	Plus/Plus

```
Query  39   ACCCGGATTCCCTTAATAA-GGCCAATGAA-AGGGAAAAAccccccccccAAACCCGGGG  96
            ||| |||||||||||| ||| ||| | |||| |||||||| ||| | || || || | ||
Sbjct  11   ACCAGGATTCCCTTAGTAACGGCGAGTGAACAGGGAAAAGCCCAGCACCGAAGCCTGTGG  70

Query  97   TCCTTTGGCCCCTAAGCAATGTGGTGTTCAGGTTAACTCCCGAAAATTCTGCTCCCCCCT  156
            || |||||||||||| ||||||||||||||||||| ||| |||| || ||||||| ||||
Sbjct  71   TCGTTTGGCCCCTAGGCAATGTGGTGTTCAGGTTAGCTCGCGAAGATGCTGCTCCACCCT  130

Query  157  AAATCCTATAATGAATAAGGTTACCCCGAAATGGCCCAATGAGGGTGAAAGGCCCCTGGG  216
            || |||||||||||| ||||||||| | || ||||||||||||||||||||||||| ||||
Sbjct  131  AAGTCCTATAATGAGTAAGGTTACTCGGACATGGCCCAATGAGGGTGAAAGGCCCGTGGG  190

Query  217  GGTGGAAATTTCAAATGGCCAATATCTTCCTGAACGGACCTTGGAATCCGGttttttttG  276
            |||||| |||    |||||||| |||||||||||| |||||||||||| || |||| ||| ||
Sbjct  191  GGTGGAGATTCAGAATGGCCAGTATCTTCCTGAGCGGACCTTGGAGTCGGGTTGTTTGTG  250

Query  277  AATGCAACCCAAAACGGGGGGTAAACTCCCTCCAAGGGTAAATACTAACCCaaatcccaa  336
            |||||| |||||| |||| |||||||||| |||||||| ||||||||| | | | ||| |
Sbjct  251  AATGCAGCCCAAAGCGGGTGGTAAACTCCATCCAAGGCTAAATACTAGCACGAGTCCGAT  310

Query  337  aacgaaaaaatataccgtgagggaaaatttaaaaatacttttaaaaaaaaaataaaaaTTGG  396
            | |||| || |||||||||||||| || ||||| |||||| || | | | |||| | | |
Sbjct  311  AGCGAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAAGAGAGAGTAAACAGTGCG  370

Query  397  TGGAAACGTTTAAAGGTAAAAAGGTGGAGTTTAACTTGAAGCTTTGAGGATTTACCTGGG  456
            || || |||| | ||||||| |||||||||| |||| ||||| |||||||| | ||||
Sbjct  371  TGAAACCGTTCAGAGGTAAACAGGTGGAGTTGAACTGCAAGCTCTGAGGATTCAGCTGGT  430

Query  457  GAATATGGCATGAACTTTGTCATATTGGTT  486
            || |||||||||| ||| ||||||||||||
Sbjct  431  GAGTATGGCATGAGCTTGGTCATATTGGTT  460
```

sample code 73

Fasciola hepatica isolate G15 28S ribosomal RNA gene, partial sequence

Sequence ID: HM369305.1 Length: 525 Number of Matches: 1

Range 1: 15 to 460 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
409 bits(453)	3e-109	356/447(80%)	1/447(0%)	Plus/Plus

```
Query  31   GGATTCCCCTTATTAACGGCAAATGAAAGGGAAAAAccccccccccAACCCGGGGGTCT  90
            |||||||| ||| |||||||| | |||| || |||| ||| | || || || | ||||
Sbjct  15   GGATTCCC-TTAGTAACGGCGAGTGAACAGGGAAAAGCCCAGCACCGAAKCCTGTGGTCA  73

Query  91   TTTGGCCCCTAGGCAATTGGGGGTTCAGGTTACCTCCCAAAAATCCTGCTCCCCCCTAAT  150
            ||||||||||||||||  || |||||||||| ||| | || || |||||||  ||||||
Sbjct  74   TTTGGCCCCTAGGCAATGTGGTGTTCAGGTTAGCTCGCGAAGATGCTGCTCCACCCTAAG  133

Query  151  TCCTTTAATGATTAAGGTTACCCGGAAATGGCCCAATGAGGGTGAAAGGCCCCGGGGGGT  210
            |||| |||||| |||||||||| |||| ||||||||||||||||||||||||  ||||||
Sbjct  134  TCCTATAATGAGTAAGGTTACTCGGACATGGCCCAATGAGGGTGAAAGGCCCGTGGGGGT  193

Query  211  GGAAATTCAAAATGGCCATTTTCTTCCTAAACAAACCTTGGATTCGGGttttttttGAAT  270
            ||| ||||| || ||||| | |||||||  | || |||||||| ||||||| ||| |||||
Sbjct  194  GGAGATTCAGAAKGGCCAGTATCTTCCTGAGCARACCTTGGAGTCGGGTTGTTTGTGAAT  253

Query  271  GCACCCCAAACCGGGGGGTAAACTCCTTCCAAGGCTAAATACTAACCCaaatccaaaacc  330
            ||| |||||| |||| |||||||||| ||||||||||||||||  | | | ||| | | |
Sbjct  254  GCASCCCAAAGCGGGTGGTAAACTCCWTCCAAGGCTAAATACTAGCACRAGTCCRATAGC  313

Query  331  aaaaaattaccgggagggaaatttaaaaattactttgaaaaaaaattaaaCATTTGTTGA  390
              || || ||||| ||||||||| || |||| |||||||||  | |  | |||||| |    |||
Sbjct  314  GAACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAARAGAGAGTAAACAGTGCGTGA  373

Query  391  AACCGTTTAAAAGTAAAAAGTTTAAGTTTAATTGCAAGTTTTAAGGTTTTACTTGGTAAT  450
            ||||||| | | ||||| || |  |||| || |||||| | | ||| || |  |||| |
Sbjct  374  AACCGTTCARAGGTAAACAGGTGGAGTTGAACTGCAAGCTCTGAGGATTCASCTGGTGAG  433

Query  451  TATGGCATAACCTTGGTCTAATTGGTT  477
            |||||||| | |||||||  |||||||
Sbjct  434  TATGGCATGAGCTTGGTCATATTGGTT  460
```

Table (1): BLASTn identification of *Fasciola* spp. based on sequence analysis.

Sample ID	Top Match Organism	Accession No.	Identity (%)	Score (bits)	E-value
4_ISF	*Fasciola* sp. isolate S10	OR676767.1	80%	388	1e−102
19_ISF	*Fasciola hepatica* isolate G15	HM369305.1	82%	456	2e−123
50_ISF	*Fasciola gigantica* isolate KhuzBufa2G	AB674553.1	83%	453	8e−123
73_ISF	*Fasciola hepatica* isolate G15	HM369305.1	80%	409	3e−109

The obtained 28S rDNA sequences from four PCR-positive samples (4_ISF, 19_ISF, 50_ISF, and 73_ISF) were analyzed using BLASTn against the NCBI nucleotide database. The results confirmed that all sequences belonged to the genus *Fasciola*, with varying degrees of identity.

Sample 4_ISF showed 80% identity with *Fasciola* sp. isolate S10 (GenBank: OR676767.1), confirming the genus-level classification but with possible sequence variability or subspecies representation.

Sample 19_ISF showed 82% identity with *Fasciola hepatica* isolate G15 (GenBank: HM369305.1) with a score of 456 bits and an E-value of 2e−123. Similarly, sample 73_ISF matched *Fasciola hepatica* (HM369305.1) with 80% identity, while sample 50_ISF aligned most closely with *Fasciola gigantica* (GenBank: AB674553.1) at 83% identity, suggesting species-level differentiation.

These results demonstrate the existence of both *F. hepatica* and *F. gigantica* lineages in the examined samples and validate the specificity of the 28S rDNA target region for molecular detection of *Fasciola* species.

Four *Fasciola* samples (4_ISF, 19_ISF, 50_ISF, and 73_ISF) were compared to the reference sequence *Fasciola hepatica* isolate Gymnocephalus cercaria (GenBank: PV082153.1) using multiple sequence alignment of the 28S rDNA region. High levels of sequence conservation were found throughout most regions in the alignment, particularly in samples 19_ISF and 73_ISF. Samples 4_ISF and 50_ISF, on the other hand, showed several nucleotide changes, suggesting species-level variation. The identification of *F. hepatica*, *F. gigantica*, and *Fasciola* sp. among the examined isolates is supported by these mutations, which are in line with earlier BLAST results. (Fig. 2)

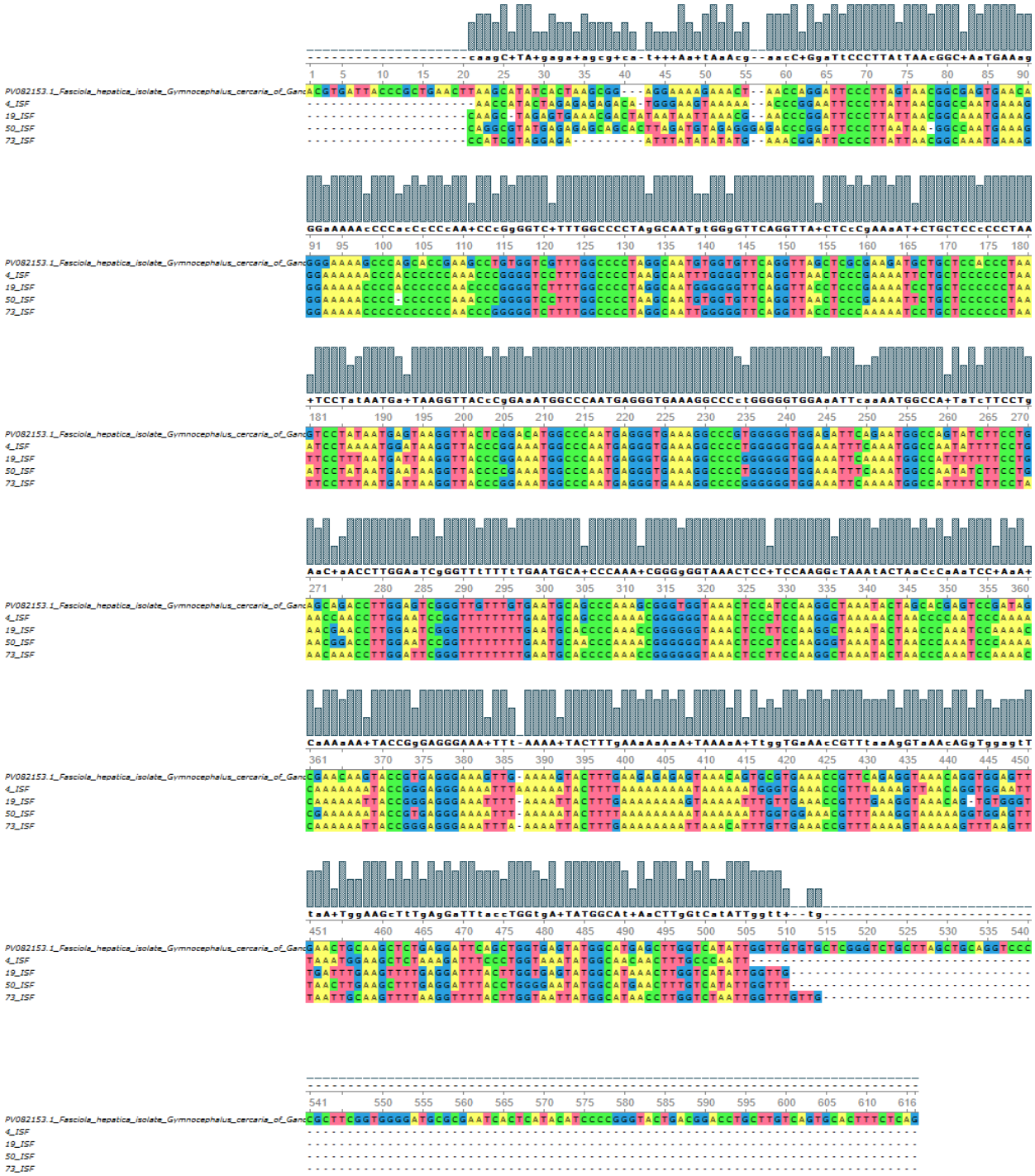

Figure 2. 28S rDNA sequences from four Fasciola isolates (45_ISF, 19_ISF, 50_ISF, and 73_ISF) were aligned many times and compared to the reference Fasciola hepatica isolate (PV082153.1). Sequence conservation or variation is indicated by colored nucleotides. High conservation is reported in most regions, whereas substitutions are detected in certain places, particularly in samples 45_ISF and 73_ISF.

To assess the evolutionary connection between the local *Fasciola* isolates and the reference sequence of *Fasciola hepatica* (GenBank: PV082153.1), a phylogenetic tree was built using the Neighbor-Joining method. All four samples were identified as *Fasciola hepatica* or closely similar variations since the tree revealed that they clustered with the reference strain. 4_ISF showed the greatest genetic distance (0.076), indicating a greater degree of diversity, whereas 19_ISF and 73_ISF showed the shortest distance (0.038). Sample 50_ISF showed a high degree of genetic similarity by closely clustering with the reference sequence. (Figure 3).

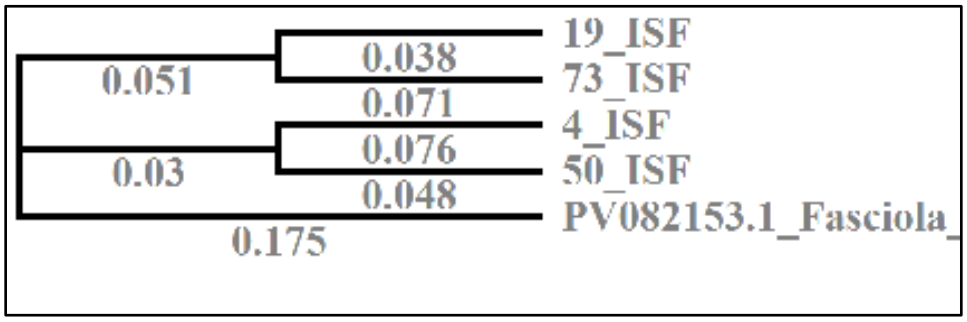

Figure 3. Neighbor-Joining phylogenetic tree of 28S rDNA gene sequences. The tree includes four local Fasciola isolates (19_ISF, 73_ISF, 4_ISF, 50_ISF) and the reference Fasciola hepatica sequence (GenBank: PV082153.1). The branches show evolutionary distances. With minor fluctuation, the tree shows genetic closeness between samples, especially in isolate 4_ISF.

The nucleotide sequences of the 28S rDNA gene from four *Fasciola* isolates were translated into amino acid sequences and aligned with the translated reference gene of *Fasciola hepatica* isolate Gymnocephalus cercaria (GenBank: PV082153.1). The translated alignment showed several conserved amino acid regions among all samples, indicating functional similarity. However, amino acid substitutions were observed at various positions, particularly in samples 4_ISF and 73_ISF, suggesting the presence of strain-level or species-level polymorphisms. These variations may reflect functional divergence or evolutionary adaptation. (Figure 4)

Figure 4. Multiple sequence alignment of translated 28S rDNA amino acid sequences from four *Fasciola* isolates and the reference sequence *Fasciola hepatica* (PV082153.1). Conserved regions are indicated by identical amino acids across samples, while substitutions highlight possible functional or evolutionary differences.

Discussion

Genetic specialization of *Fasciola* species based on a large subunit gene of 28S ribosomal DNA provides significant analysis in terms of genetic variability of Iraqi *Fasciola* species. These study results provide a significant specificity of primers that trigger the targeted genes, giving more precise results along with ribosomal DNA of the parasite's fluke (12,13). In the Middle East regions, the BLAST analysis showed different identity levels about *F. hepatica* isolates, whereas it showed very high similarity about *F. gigantica*, these results interpret a group distribution of the 2 species in the epidemic regions (14,15). In previous genetic studies, the nucleotide substitution level of 45 ISF and 73 ISF was reported for intraspecific hybridisation and variability (16,17). The presence of a very high conservation degree of 28s ribosomal DNA gene, mainly between *Fasciola hepatica* isolates, as well as polymorphism was significant in certain gene sequences reporting intraspecies variation level, there are a cluster of isolates with phylogenetic analysis about *Fasciola hepatica* of a reference gene, whereas the presence of a few co-existence of a distinct divergent genetically lineages (14,16). The sequence of amino acid analysis showed a highly conserved domain of protein constructing a physicochemically conserved ribosomal subunit, in addition to a small level of genetic substitution in some isolates, reporting mechanisms of adaptation to evolution that affect a parasite's habit, host shelter specificity and treatment sensitivity (18,19). Finally, our results regarding genetic variation and intraspecific complexity of *Fasciola* studied species revealed the essential surveillance and investigations at the genetic level, focusing on the hybridization monitoring and species differentiation. Effective measurements to parasitic infection are considered a good strategy to control epidemiology, pathogenicity and outcome of treatments (17,20).

Conclusions

The molecular results of this study targeting 28S rDNA of *Fasciola* species isolates revealed the genetic variation and coexistence of the two species with genetically evolutionary divergent strains. The primers of this study were very species-specific and efficient for targeting the amplification of this gene. The phylogenetic classification analysis showed highly conserved polymorphism with variation in strain level hybridization.

Acknowledgment

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Conflict of Interests

The authors declare that they have no potential conflict of interest with respect to the publication of this article.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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تتابع تسلسل جين الحمض النووي الريبوزي (rDNA) لجنس المتورقة في الماشية في العراق

إسماعيل وعد الله إسماعيل

قسم تقنيات التخدير، كلية التقنيات الصحية والطبية/ بغداد، الجامعة التقنية الوسطى، بغداد، العراق.

الخلاصة

داء المتورقة مرض حيواني المنشأ خطير، تسببه أنواع المتورقة التي تصيب الإنسان والحيوانات مثل الأغنام والأبقار والجاموس في جميع أنحاء العالم، مما يسبب مشاكل صحية وبيطرية عامة. ركزت هذه الدراسة على التمايز الجيني بين نوعي المتورقة (*F. gagantica, F. hepatica*) باستخدام وحدة فرعية كبيرة من جين (28S rDNA) تم عزل 45 دودة بالغة من أكباد الابقار مختلفة في محافظة البصرة العراقية، وتم استخراج الحمض النووي الجيني باستخدام مجموعة خاصة على أنواع المتورقة المعزولة مع تضمين بادئات محددة بطول 21 زوج قاعدي تستهدف (28S rDNA) ظهرت نتائج BLAST و PCR تطورًا وتنوعًا وراثيًا مع تشابه بنسبة 80٪ تقريبًا بين نوعي المتورقة. خلصت النتائج من الناحية التطورية إلى حفظ وتعدد أشكال عالية الخصوصية للأنواع مع احتمال تهجين السلالات.

الكلمات المفتاحية: *F. hepatica* ، *gigantica*, ,. تتابع, الماشية.