Infection and Drug Resistance

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ORIGINAL RESEARCH

Molecular Characterization of *Fasciola hepatica* in Sheep Based on DNA Sequences of Ribosomal ITS-1

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Introduction: World Health Organization (WHO) considers Fascioliasis as a neglected tropical disease that requires global efforts for disease control. Data from the genetic characterization of *Fasciola* population shed light on the spread of infections among animals which could help in the development of effective parasite control. The aim of the present work was to genetically characterize Fasciola adult worms isolated from sheep in Saudi Arabia by sequence analysis of ITS-1 region.

Methods: A total of 12,653 slaughtered sheep in Jeddah city, Saudi Arabia were examined for the presence of *Fasciola* spp. adult worms. The ITS-1 region of all parasites was amplified and sequenced.

Results: Overall, 12 variants DNA sequences were obtained. The variance of isolates ranged from 0.00771 to 0.34405. BLAST search showed that all obtained sequences *were Fasciola hepatica* and had >99.3% similarity with *F. hepatica* isolates from Spain and USA (from different hosts other than sheep). Phylogenetic analysis showed that *Fasciola* isolates were closely related to isolates from different countries.

Discussion: The current study showed that *F. hepatica* was the only spp. isolated from sheep in Jeddah. Further studies from different localities in Saudi Arabia are needed to help in the development of disease control.

Keywords: genetic diversity, characterization, *Fasciola hepatica*, parasitic diseases, sheep

Introduction

Fascioliasis is a zoonotic disease of public health significance and a worldwide distribution.[1] The causative agents of Fascioliasis are *F. hepatica* (*F. hepatica*; temperate liver fluke) and *Fasciola gigantica* (tropical liver fluke).[2,3] Although it has been recognized for centuries, the disease is currently expanding and has a serious impact on humans, animals, and livestock all over the world.[4,5] It is well documented that fasciolosis, according to the World Health Organization (WHO), is a tropical zoonotic disease with public health concern which necessitate effective disease control and prevention measures.[6] The primary definitive hosts of *Fasciola* species are sheep and cattle, with hundreds of millions of animals are estimated to be infected.[7,8] A wide range of symptoms have been reported in animal fascioliasis. Within 8–10 weeks of infection, severe anemia, liver failure, and mortality may occur.[9–12] The disease usually reduces the animal

Received: 13 May 2023
Accepted: 25 July 2023
Published: 12 October 2023

production of meat, milk, and wool and increases the likelihood of secondary bacterial infections.[13] Animal fascioliasis results in an annual economic loss of more than 3.2 million dollars worldwide.[14] Although fascioliasis is primarily a disease of ruminants, outbreaks of human infections have been documented during the past three decades. Fascioliasis is reportedly reemerging and emerging in some Middle Eastern, African, and Asian nations.[15–19] Several European nations, including Portugal, Spain, and France, continue to record cases of locally acquired human fascioliasis.[18,19] Infection is rare in North America, where most of the recorded cases are immigrants and visitors.[20–23]

Estimates indicated that 17 million individuals are infected and 180 million are at risk of infection.[4] Children are the most commonly affected by fascioliasis, especially in low-income rural areas in South Asia, Africa, and Asia.[12] Animal fascioliasis has been reported in Saudi Arabia where prevalence rates for sheep and cattle are 13.5% and 52.9%, respectively.[13,24] In Saudi Arabia, fascioliasis is thought to be the primary cause of liver condemnation of 52.06% of cattle, causing an annual economic loss of $0.3 million dollars.[13] Additionally, human cases of fascioliasis among foreign workers in Saudi Arabia have been also reported.[25]

It is often difficult to differentiate between *Fasciola* species in epidemiological studies on the basis of the morphological characteristics of the parasite.[26] So, following morphological identification to genus level, molecular approaches are usually applied for the genotyping of the *Fasciola* parasite. DNA-based methods for amplification and sequencing of nuclear ribosomal internal transcribed spacers (ITS-1, ITS-2), 28S rRNA, mitochondrial NADH: Ubiquinone Oxidoreductase Core Subunit 1 (MT-ND1), and cytochrome c oxidase I (COI) genes are commonly used for genetic characterization.[27–30] In both liver fluke taxonomy and epidemiology, these methods are often used.[31,32] The distribution and spread of infection among animals are revealed by data from the genetic characterization of the *Fasciola* population, which may one day aid in the creation of efficient parasite control strategies and parasite elimination.[31,32] Furthermore, genetic research is essential to determining the origin, toxicity, evolution, and development of parasites' anthelmintic treatment resistance.[33] There is not much data for the genotyping of *Fasciola* spp. in Saudi Arabia, despite the fact that extensive DNA sequencing for *F. hepatica* and *F. gigantica* is available from numerous nations. The goal of the current work was to use sequence analysis of the internal transcribed spacer (ITS-1) region to genetically characterize *Fasciola* spp. isolated from sheep in Jeddah, Saudi Arabia.

Materials and Methods

Study Area

During the first of June and the first of July of 2020, the current study was conducted in Jeddah, Saudi Arabia. A Saudi city called Jeddah is located in the middle of the Red Sea's eastern shore. Jeddah has a warm climate in the winter compared with the other Saudi Arabian towns, with temperatures ranging from 15 °C in the morning to 28 °C in the afternoon. Summers are so hot and humid, especially in September. Jeddah experiences limited rainfall, with the most of it falling in November and December.

Sample Collection and Parasitological Analysis

A total of 12,653 slaughtered sheep in Jeddah's central municipal abattoir were subjected to the present study. Animals included both males and females of two age groups; young (less than one year) and adults (more than one year). Livers and gall bladders of slaughtered animals were examined for the presence of adult worms of *Fasciola* spp. The collected worms were identified initially on the basis of morphological characteristics using a dissecting microscope,[34] after which they were washed extensively in phosphate buffer saline (PBS) and kept in 80% ethanol at room temperature until further use for extraction of genomic DNA.

After complete evaporation of ethanol, each sample was washed in distilled water for 3 consecutive times then subjected to genomic DNA extraction. A small part of the parasite tissue was removed from the lateral zone of adult flukes (to avoid likely contamination by sperm or eggs present in the reproductive organs) and was used for DNA extraction and crushed into tiny particles.[34] Total genomic DNA extraction was done using the Jena Bioscience Blood-Animal-Plant DNA Preparation – Columns Kit (250 preparations) (Catalog Number: PP-213) following the manufacturer's protocols, and stored at −20°C until further use. Primers targeting the 680 bp internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA (rRNA) genes were used for Polymerase Chain Reaction (PCR) (Table 1).

Table 1 Showing Primers Targeting Internal Transcribed Spacer-1 (ITS-1) Region of *Fasciola* Adult Worms

Gene Primer	Sequence	Expected Weight	Reference
ITS-1F	5'-TTG CGC TGA TTA CGT CCC TG-3		
ITS-1R	5'-TTG GCT GCG CTC TTC ATC GAC-3'	680 bp	[22]

PCR reaction with a total volume of 50 µL contains 5 µL of DNA solution, 25 µL liters of mastermix 2X (Thermo Fisher Scientific, USA), 1.0 µL primer (0.2 M), and 18.0 µL of distilled water was used. The following reaction conditions were used for PCR in a thermal cycler (Master-cycler Personal, Eppendorf): pre-denaturation for 10 min at 95 °C, then 25 PCR cycles of denaturation at 94 °C for 90 seconds, annealing at 58 °C for, and elongation at 72 °C for 90 seconds, followed by a final extension of at 72 °C for 10 min. Through electrophoresis on a 1% agarose gel with TAE buffer containing SYBR Safe (Invitrogen, SYBR SafeTM, Cat. No. S33102), PCR products were examined. For 90 minutes, electrophoresis was carried out at 90V. A GelDoc EZ Imager was used to report the size of the PCR products (Bio-Rad, GelDoc EZ Imager, Cat. No. 1708270).

Nucleotide Sequences of the ITS-1 Gene

To confirm the PCR results and identify the species of *Fasciola* adult flukes isolated from infected sheep in the present study, the obtained PCR products were used as templates for DNA sequence. Amplified ITS-1 fragments were sent to Macrogen (Seoul – South Korea) (https://dna.macrogen.com/) for sequencing of the ITS-1 region using the same primers used for PCR.

Sequence and Phylogenetic Analyses

NCBI BLAST search (with default settings) was used to retrieve related *Fasciola sp*. sequences from the GenBank. The obtained DNA sequences were aligned against DNA sequences of *Fasciola* species from different hosts and countries that had previously been deposited in the GenBank. Multiple alignments were done using the MUSCLE program of MEGA X software[35]. All sequences obtained from the current study (12 unique sequences) were deposited in the GenBank and as-signed the accession numbers from MZ429416.1 to MZ429427.1. Using the Tamura 3 parameter model of the MEGA X program and the Maximum Likelihood approach (ML), phylogenetic analysis and pairwise nucleotide changes of the ITS-1 region were performed. *Fascioloides magna* was used as outgroup (accession no. EF534991). Sequences of *F. hepatica* from different countries used for construction of the phylogenic tree together with sequences obtained from the present study are shown in Table 2.

Statistical Analysis

The gathered data and outcomes were statistically analyzed using the SPSS version 22 program (Statistical Program for the Social Sciences).[42] The Chi-square test was used to analyze the category variables. Statistical significance was defined as a P-value of 0.05 or lower.

Results

Prevalence of *Fasciola* Infection in Sheep

The present study included both local and imported Somalian animals (42.93% versus 57.07%). Examination of the liver and gall bladder of 12,653 slaughtered sheep revealed that 1043 (8.24%) were infected with *Fasciola* adult worm, 7.16% were local sheep and 9.6% were imported animals (Table 3).

Significantly higher infection rate was reported among adults than young animals (14.27% vs 1.46%; P = 0.002). Moreover, female animals were recognized to have higher infection rates than males with no statistical significance (16.69% vs 2.6%, P = 0.279) (Table 4).

Table 2 Showing Accession Numbers of *Fasciola* spp. Used for Construction of the Phylogenetic Tree

	Accession Number	Country	Reference
1.	MZ429416.1	Current study	
2.	MZ429417.1	Current study	
3.	MZ429418.1	Current study	
4.	MZ429419.1	Current study	
5.	MZ429420.1	Current study	
6.	MZ429421.1	Current study	
7.	MZ429422.1	Current study	
8.	MZ429423.1	Current study	
9.	MZ429424.1	Current study	
10.	MZ429425.1	Current study	
11.	MZ429426.1	Current study	
12.	MZ429427.1	Current study	
13.	JF824666.1	Italy	[36]
14.	AM707030.1	Andorra	[37]
15.	AB553690.1	Egypt	[38]
16.	JF708028.1	China	Chen (2011) Direct submission
17.	JN828959.1	Iran	[39]
18.	JF432078.1	Iran	[39]
19.	JF708031.1	USA	Chen (2011) Direct submission
20.	HE972273.1	Saudi Arabia	[40]
21.	JF708034.1	France	Chen (2011) Direct submission
22.	JN828960.1	Iran	[39]
23.	JF432072.1	Iran	[39]
24.	JF432076.1	Iran	[39]
25.	HM746786.1	Iran	[39]
26.	HM746785.1	Iran	[39]
27.	EF534991.1	*Fasciolid magna*	[41]

Table 3 Infection Rate Among Local and Imported Animals in the Present Study

	Examined Animals	Infected Animals
Local animals	5432 (42.93%)	389 (7.16%)
Imported animals	7221 (57.07%)	654 (9.6%)

Table 4 Gender and Age Group of *F. hepatica*-Infected Sheep. The Numbers (N) of Infected Male, Female, Adult, and Young Sheep Were Determined and Percentages (%) of Total Infected Animals Were Calculated

Category		Total Examined (*N*=12653)	Infected (*N*=1043)	*P*-value
Sex	**Male** [*N* (%)]	7589 (59.9%)	198 (2.60%)	0.279
	Female [*N* (%)]	5064 (40.1%)	845 (16.69%)	
Age	**Young** [*N* (%)]	5952 (47%)	87 (1.46%)	0.002
	Adult [*N* (%)]	6701 (53%)	956 (14.27%)	

PCR Amplification of *F. hepatica* ITS-1 Gene

Fasciola spp. adult worms were collected from infected animals, then genomic DNA was extracted and successfully amplified from all examined adults (1043). PCR products of 680 bp were obtained (Figure 1).

Next, all PCR products (n = 1043) were used for DNA sequence of the ITS-1 region. Twelve different DNA sequences were obtained. The obtained nucleotide sequence data were deposited in the GenBank (Accession numbers MZ429416.1 - MZ429427.1). The variance of isolates ranged from 0. 00771 to 0.34405. Detailed results of nucleotide variation are shown in Table 5.

Using the BLAST 2 software, all sequences obtained from the present study were aligned with *F. hepatica* sequences deposited in the GenBank from different countries. DNA sequences of the present study shared >99.3% identity with *F. hepatica* sequences from Iran (MK377136, MF969010) and Egypt (LC076196).

The Maximum Likelihood method and Tamura-3 model were used to construct a phylogenetic tree to compare the DNA sequences of the present study with other *F. hepatica* Gen-Bank-accessible sequences. The phylogenetic tree supported the results of BLAST research as the isolates from the present work were closely related to *F. hepatica* isolates deposited in the GenBank. *F. hepatica* isolates were arranged in two clusters. Figure 2 displays a trustworthy grouping of the ITS-1 sequences of *F. hepatica* from the current investigation with those from Iran, Asia (China and Saudi Arabia), Europe (Italy and France), Africa (Egypt and Andorra), and the USA.

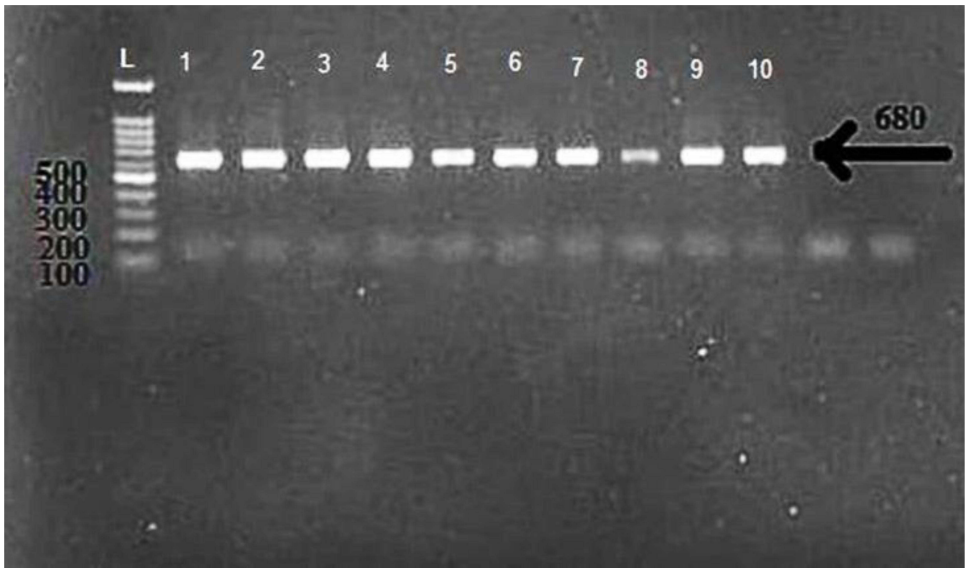
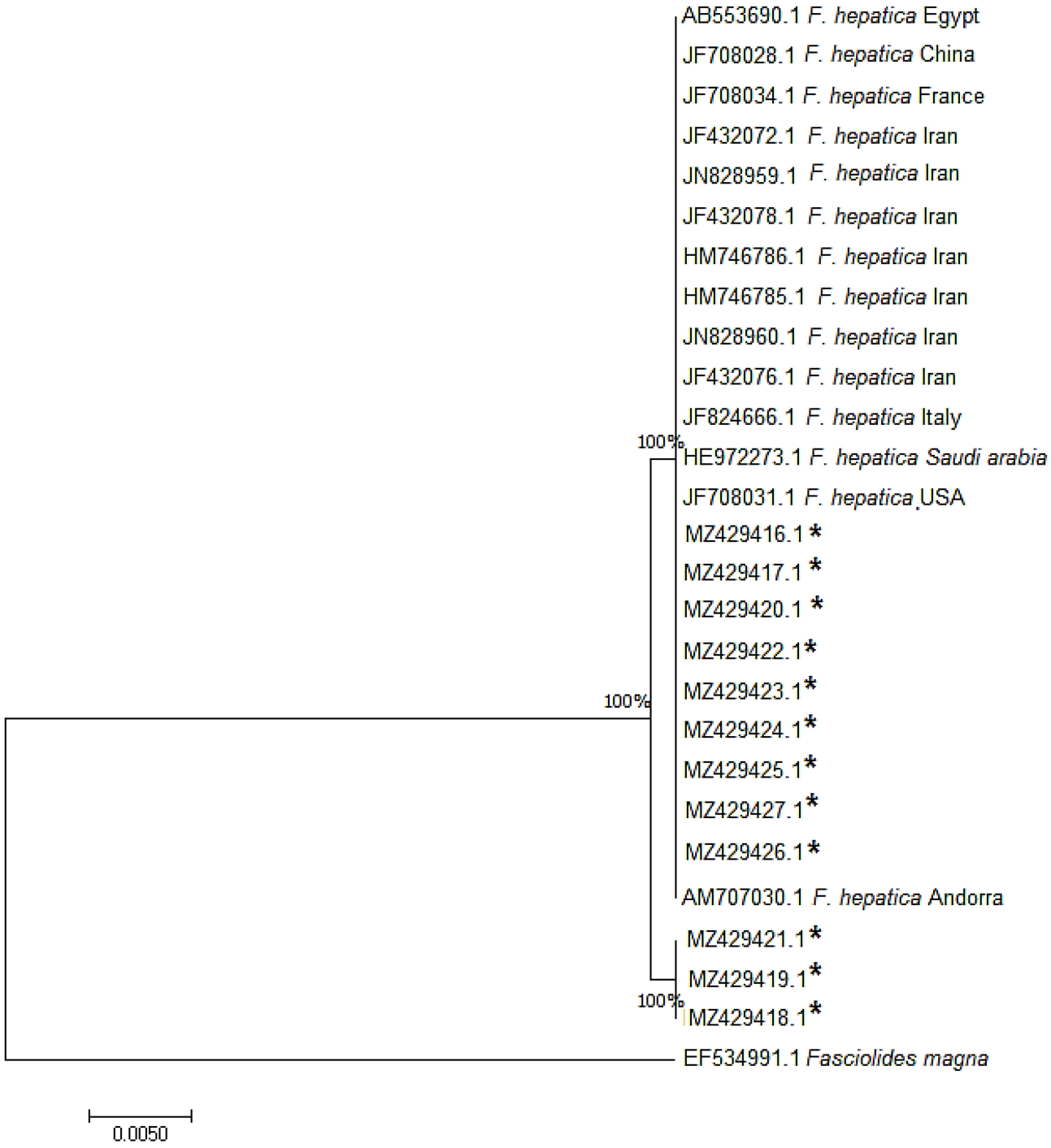


Figure 1 Agarose gel electrophoretic analysis of representative ITS-1 PCR products (680 bp) of *F. hepatica* adult worms. Lane of 1–10 representing samples. L: DNA ladder (100bp).

Table 5 Genetic p-Distances Between Haplotypes Based on Sequence Analysis of Partial ITS1 Gene Analysis of *F. hepatica* Samples Obtained in the Present Study

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
H1												
H2	0.02063											
H3	0.03041	0.04440										
H4	0.02390	0.04276	0.01392									
H5	0.02541	0.03950	0.01394	0.01236								
H6	0.01899	0.03452	0.01863	0.01080	0.01389							
H7	0.02708	0.04109	0.01239	0.00771	0.01543	0.11147						
H8	0.01891	0.03937	0.02178	0.01236	0.02320	0.11580	0.26185					
H9	0.03345	0.03939	0.02022	0.01394	0.02167	0.15608	0.34405	0.34168				
H10	0.03174	0.04421	0.02657	0.01866	0.02649	0.01702	0.01861	0.01850	0.02335			
H11	0.03026	0.04751	0.02019	0.01862	0.01856	0.01387	0.01857	0.01851	0.02488	0.02323		
H12	0.02859	0.04112	0.01242	0.00772	0.01854	0.01702	0.01386	0.01381	0.02018	0.01851	0.01857	

https://doi.org/10.2147/IDR.S421206 DovePress



*Indicates sequences of the present study.

Figure 2 Phylogenetic relationship of *F. hepatica* isolated from Saudi Arabian sheep to other *Fasciola* sp. deposited in the GenBank based on the partial (ITS-1) gene nucleotide sequences, using *Fascioloides magna* as an out-group trematode.

Discussion

Animal fascioliasis is usually a subclinical disease that is associated with economic losses, mostly due to a decline in animal productivity and liver condemnations.[43,44] So, global efforts for disease control and prevention are needed to minimize the impact of the disease on animals and livestock. A deeper understanding of the dynamics of disease epidemiology and parasite

taxonomy may be gained through advanced molecular and genetic studied and could aid in the development of effective control programs.[31,32] In the present study, we examined 12,653 slaughtered sheep at the municipal abattoir of Jeddah, Saudi Arabia. We found that 8.2% of sheep were infected. Previous findings from different localities in Saudi Arabia reported a wide range of infection rates. Higher infection rate was reported in Riyadh by Magda and Al-Megrin in 2005 (21.9%)[24] and by Degheidy et al in 2012 (16.9%).[13] On the other hand, lower prevalence had been reported by Mgzoub and Kasim who examined different animals from different regions in Saudi Arabia and reported an infection rate in sheep ranging from 0.18% to 2.4%.[45] Different infection rates from different localities may be explained by several factors including climate, ecological conditions, seasons, sources, and types of animals which may play a role in this issue.

In the present study, higher prevalence of fasciolosis was found among adults with statistical significance. In agreement with this finding, previous studies reported higher infection rates among adults than young animals.[46,47] On the other hand, regarding gender, female sheep showed higher infection rate than males (with no statistical significance). This finding is supported by results of previous studies indicating the difference in susceptibility of both sexes to fasciolosis.[46,48,49] This difference may be attributed to exposure of females to stress during pregnancy and parturition increasing their probability to infection.[50]

In the present study, PCR and DNA sequence were used to further identify and characterized Fasciola isolates. Several genetic loci including ITS regions (ITS-1 and ITS-2) have been used as a good target for diagnosis and genetic characterization of *Fasciola* spp.[6,30,31,34,38,44,51–56] We used primers targeting 680 bp ITS-1 region. The obtained DNA sequence data confirmed that the isolated *Fasciola* adult worms are *F. hepatica*. This finding is in agreement with previous results reported by Alajmi, who studied Fasciola population isolated from sheep in Riyadh, Saudi Arabia.[28] The author reported infection of sheep with both *F. hepatica* and *F. gigantica* with predominance of *F. hepatica* (80%).[28] Furthermore, in the Al Taif region of Saudi Arabia, Shalaby et al reported identical infection rates for both *F. hepatica* and *F. gigantica* in imported sheep.[40] *Fasciola hepatica* is the most widespread and common species in temperate regions, while *F. gigantica* is found in tropical countries of Africa, according to numerous studies based on both nuclear and mitochondrial sequences.[57–61] Several studies particularly in Asian populations reported pure and mixed forms of *Fasciola* species in ruminants; Japan,[31,62] China,[63,64] Korea,[31,57,65] Iran,[66,67] Bangladesh,[68] and Vietnam.[69,70] The phylogenetic analysis of ITS-1 sequences using MEGA X application showed a rooted tree with *Fasciolid magnua* ITS-1 as an outgroup. The 12 Saudi Arabian *F. hepatica* isolates have been grouped into two clades with isolates from Iran, Africa (Egypt and Andorra), Asia (China and Saudi Arabia), Europe (Italy and France), and the USA.

Conclusion

Our research revealed that the only *Fasciola* species living in sheep included in this paper is *F. hepatica*. We think that by better comprehending the distribution and spread of *Fasciola* species among animals in Saudi Arabia, we can create more effective management strategies. It is well known that using molecular methods is the best way to distinguish between distinct *Fasciola* species based on several genetic loci. Even though there have been numerous reports of *Fasciola* infections in Saudi Arabia, there has only been a limited amount of molecular evidence to support any of these findings. To give information that will aid in the creation of fascioliasis management strategies and reduce economic losses, additional research utilizing a sizable number of samples from various locations in Saudi Arabia are required.

Ethics Statement

The present study was approved by the Institutional Review Board, College of Science, University of Jeddah, Saudi Arabia (protocol code UJ212430061).

Funding

This research was supported by Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2023R39), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia and by the Researchers Supporting Project number (RSP2023R25), King Saud University, Riyadh, Saudi Arabia.

Disclosure

The authors report no conflicts of interest in this work.

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