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ORIGINAL RESEARCH

Identification and Genetic Characterization of *Fasciola hepatica* Isolated from Cattle in Jeddah, Saudi Arabia Based on Sequence Analysis of Mitochondrial (COI) Gene

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Background: In Saudi Arabia, more than US$ 0.2 million annual losses are caused by liver condemnations due to fascioliasis. Data obtained from the genetic characterization of *Fasciola* population sheds light on parasite transmission which could eventually help in development of effective parasite control measures. So, the aim of this study was to investigate the genetic diversity of *Fasciola* spp. isolated from cattle in Saudi Arabia by sequence analyses of COI gene.

Materials and Methods: A total of 325 cows slaughtered at the central municipal abattoir in Jeddah city, Jeddah Province, Saudi Arabia were examined for fascioliasis in the period from 1st of June to 1st of July 2020. DNA was extracted from adult *Fasciola* worms and used for PCR and DNA sequence using a primer pair targeting COI gene. Analysis of the obtained sequences was done using BLAST search and phylogenetic analysis.

Results: Bovine fascioliasis was diagnosed in 18 out of 325 cattle (5.5%). Forty-eight flukes were extracted from infected animals and DNA was successfully amplified from all flukes. Overall 12 different DNA sequences were obtained. BLAST search showed that all obtained sequences were *F. hepatica* and had >97% similarity with *F. hepatica* isolates from Tanzania, Europe and Iran. Phylogenetic analysis of the obtained sequences showed that *Fasciola* isolates from the current study were clustered in one subclade closely related to isolates from North and South Africa and Italy.

Conclusion: Reports on the molecular characterization of *Fasciola* spp. in Saudi Arabia are limited. In the current study, our findings showed that *F. hepatica* was the only *Fasciola* species parasitizing cattle in Jeddah city, Saudi Arabia. Further studies using a large number of samples from different localities in Saudi Arabia are needed to provide data that will help the development of control measures against fascioliasis.

Keywords: fascioliasis, cattle, PCR, COI, Saudi Arabia

Introduction

Fascioliasis is a zoonotic helminthic infection caused by *Fasciola hepatica* and *F. gigantica*.[1] Fascioliasis causes serious economic and health problems; especially in developing countries, due to high cost of anthelmintic drugs.[2]

The definitive host acquire fascioliasis by ingestion of metacercariae. The disease usually results in decreased animal production of meat, milk, and wool as well as a higher prevalence of secondary bacterial infections, with an annual

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economic loss of more than 2000 million dollars worldwide.[3] Triclabendazole has long been used as the first line of treatment for fascioliasis, due to its therapeutic efficacy against all developmental stages of the parasite.[4] However, emerging resistance has been reported to this drug, which complicates the treatment of fascioliasis in farmed ruminants.[4]

Although, fascioliasis is primarily a disease of ruminants (sheep and cattle), outbreaks of human infections have been reported in the last three decades.[5] It was estimated that 118 million humans are infected while 180 million are at risk.[6] In the past, the disease was restricted to specific geographical areas; however, according to the World Health Organization (WHO), human cases are increasingly reported in Europe, North and South America, Oceania, Africa and Asia.[7]

In Saudi Arabia, animal fascioliasis has been reported with prevalence of 13.5% and 52.9% in sheep and cattle respectively.[8–10] Fascioliasis is considered the main cause of total liver condemnation in 52.06% of cattle in Saudi Arabia, whereas meat and offal's condemnation resulted in economic loss of 0.2 million dollars annually.[8,9] Moreover, human cases of fascioliasis were reported among immigrant workers in Saudi Arabia.[8,11]

In epidemiological studies, it is usually difficult to differentiate accurately between *Fasciola* species based on morphological criteria.[12] So, following morphological identification, molecular approaches have been applied for the genotyping of the *Fasciola* parasite such as random amplified polymorphic DNA (RAPD),[12] single nucleotide polymorphism (SNP),[13] polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)[14] and sequencing of the whole genome.[15,16]

Data obtained from the genetic characterization of *Fasciola* population sheds light on the distribution and spread of infections among animals which could eventually help in development of effective parasite control measures and parasite elimination.[15,17] Moreover, genetic studies are critical to find the source, infectivity, pathogenicity, evolution and development of anthelmintic drug resistance in parasites.[18]

Although significant DNA sequencing for *F. hepatica* and *F. gigantica* are available from several regions, there is few data for the genotyping of *Fasciola* spp. in Saudi Arabia. Thus, in the present study, we aimed – for the first time – to genetically characterize *Fasciola* spp. isolated from cattle by sequence analyses of the mitochondrial cytochrome oxidase subunit 1 gene (COI).

Materials and Methods

Study Area

The current research was conducted in Jeddah city, Jeddah Province, Saudi Arabia, between 1st of June and 1st of July 2020. Jeddah city is a Saudi city located on the eastern bank of the Red Sea. In contrast to other Saudi Arabian cities, Jeddah has a warm winter climate, with temperatures ranging from 15°C in the morning to 28°C in the afternoon. Summers are quite hot and humid, specifically in September. The rainfall in Jeddah is short, with the majority of it falling in November and December (Figure 1).[19]

Parasites Collection

A total of 325 cows, slaughtered in Jeddah Municipal abattoir, were divided into two groups (young <1 year and adults >1 year old) and included in the present study. The liver and gall bladders of slaughtered cows were examined for the presence of *Fasciola* spp. adult worms. Identification of the collected parasites was done using the dissecting microscope on basis of their morphological features.[20] All live worms were collected and incubated in warm RPMI 1640 media (Gibco, Life Technologies, CA, USA; Catalog number: 11875093) for 5 h at 37°C to allow regurgitation to prevent contamination by host blood present in the worm gut. The media was changed 3–5 times during incubation. Collected worms were then washed with saline and transferred into sterile containers with 70% ethanol and refrigerated at 4°C for later use.[21]

DNA Extraction

A small piece of the apical zone of the posterior end of adult worms (n = 48 isolated from 18 infected cows; two to four worms from each cow) was cut to remove likely contamination by sperm or eggs present in the reproductive organs and was used for DNA extraction.[22] This piece was then homogenized using a micro-electric tissue homogenizer and

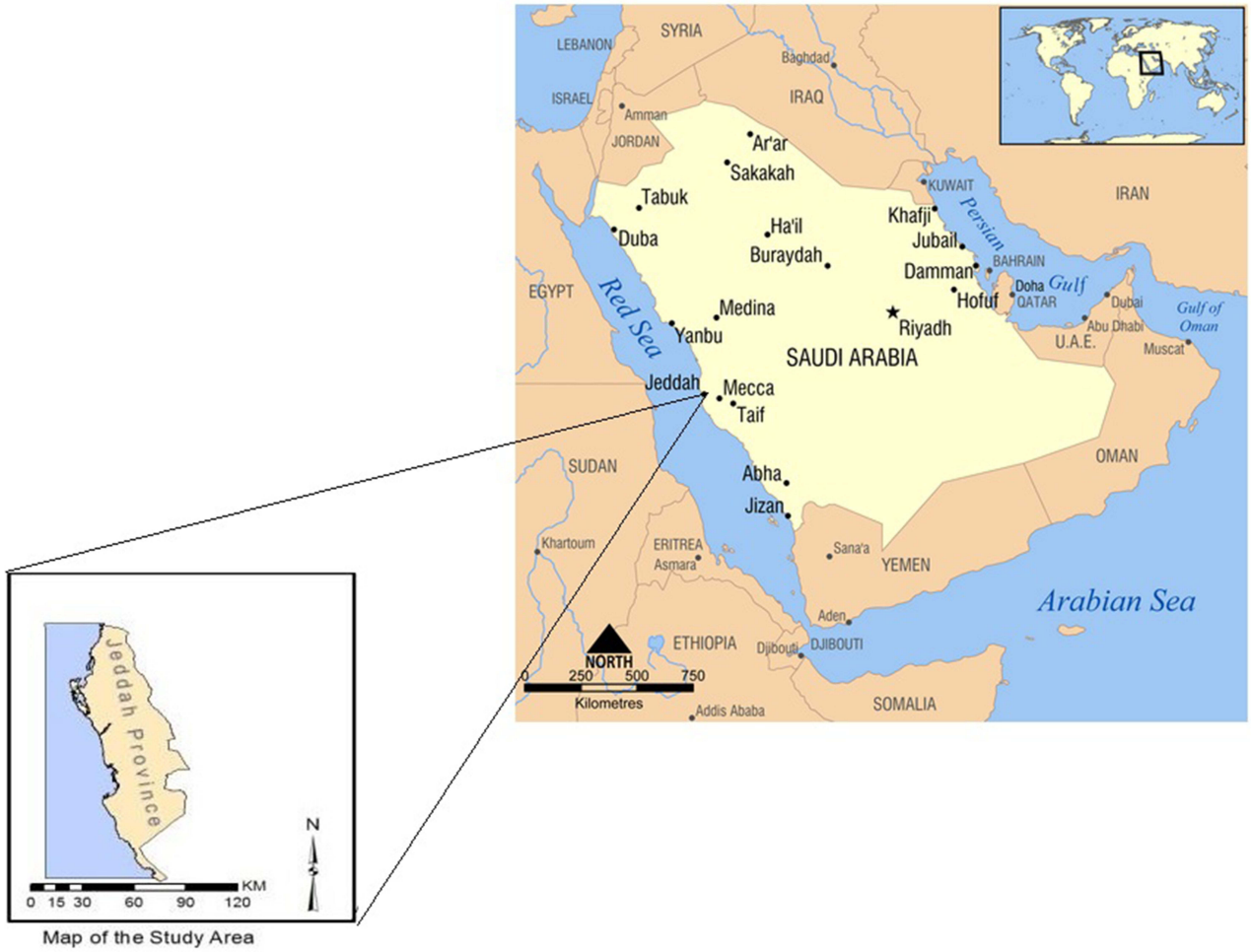

Figure 1 A map showing jeddah province in Saudi Arabia (the study area). Redrawn from https://www.wikiwand.com/en/List_of_cities_and_towns_in_Saudi_Arabia.[19]

genomic DNA was obtained using the DNeasy Blood & Tissue DNA extraction kit (Qiagen, Germantown, MD, USA, Cat. No./ID: 69504), based on the guidelines of producer.

Polymerase Chain Reaction (PCR)

PCR reaction, in a total volume of 50 µL, was performed using thermal cycler (Z316091 Eppendorf® Mastercycler Personal, AC/DC input 230 V AC, 50–60 Hz) to amplify the partial COI gene. Two primers, Ita8 (forward: 5′-ACGTTGGATCATAAGCGTGT-3′) and Ita9 (reverse: 5′-CCTCATCCAACATAACCTCT-3′) were used according to previous studies.[23] The PCR conditions were set as following: initial denaturation at 95°C for 5 min, 36 cycles of denaturation at 95°C for 40 seconds, annealing at 46°C for 55 seconds and extension at 72°C for 1 min. Final extension was done for 10 min at 72°C. The predictable size of the PCR product was 493 bp.

PCR products were analyzed by electrophoresis on a 1% agarose gel (Bioline, Cat. No. BIO-41027) in TAE buffer containing SYBR Safe (Invitrogen, SYBR Safe™, Cat. No. S33102). Electrophoresis was performed at 90V for 90 min. PCR products size was reported by using a GelDoc EZ Imager (Bio-Rad, GelDoc EZ Imager, Cat. No. 1708270).

DNA Sequencing

PCR products (48 adult worms extracted from 18 infected cows) were purified using the Bioline Isolate PCR and Gel kit (London, UK, Isolate II PCR and Gel kit Cat. No. BIO-52059) following the manufacturer's instructions. PCR products were then sequenced using the same forward and reverse primers used for PCR.

Sequence and Phylogenetic Analyses

Obtained sequences of PCR products were aligned with DNA sequences of *Fasciola spp*. deposited from different countries in the GenBank using BLAST software. Multiple alignments were done using MUSCLE program of MEGA X software.

Phylogenetic analysis and Pairwise nucleotide variations of COI gene were performed using the Maximum Likelihood method and Tamura 3 parameter model of the MEGA X software. Sequences used for construction of the phylogenetic tree are listed in Table 1.

Statistical Analysis

All obtained data and results were statistically analyzed by SPSS program (version 22). Differences between groups were determined using Chi-square. P values < 0.05 were considered statistically significant.

Ethical Statement

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

Results

Examination of the livers and gall bladders of 325 cows slaughtered at the central Municipal abattoir in Jeddah city, revealed that 18 cows (5.5%) were infected with *Fasciola* adult worms (Figure 2). Higher infection rates were reported

Table 1 Shows the Sequences Used for Construction of the Phylogenetic Tree

	Accession Number	Country	Reference
1.	JF824674.1	Italy	[33]
2.	KT182261.1	South Africa	[34]
3.	AB207170.1	Uruguay	[23]
4.	AB553828.1	Egypt	[36]
5.	AB553823.1	Egypt	[36]
6.	AB553810.1	Egypt	[36]
7.	GQ121276.1	Turkey	[14]
8.	AB207103.1	Australia	[23]
9.	GQ231550.1	Tunisia	[28]
10.	GQ398052.1	Iran	[37]
11.	GQ398053.1	Iran	[37]
12.	GQ398054.1	Iran	[37]
13.	MK212144.1	Algeria	[35]
14.	GQ231551.1	North Africa	[28]
15.	MN527593.1	Iran	[38]
16.	KR422388.1	Poland	[39]
17.	MN527595.1	Iran	[38]
18.	MN527594.1	Iran	[38]
19.	MN527597.1	Iran	[38]
20.	AF040935.1	*Paragonimus westermani*	[40]

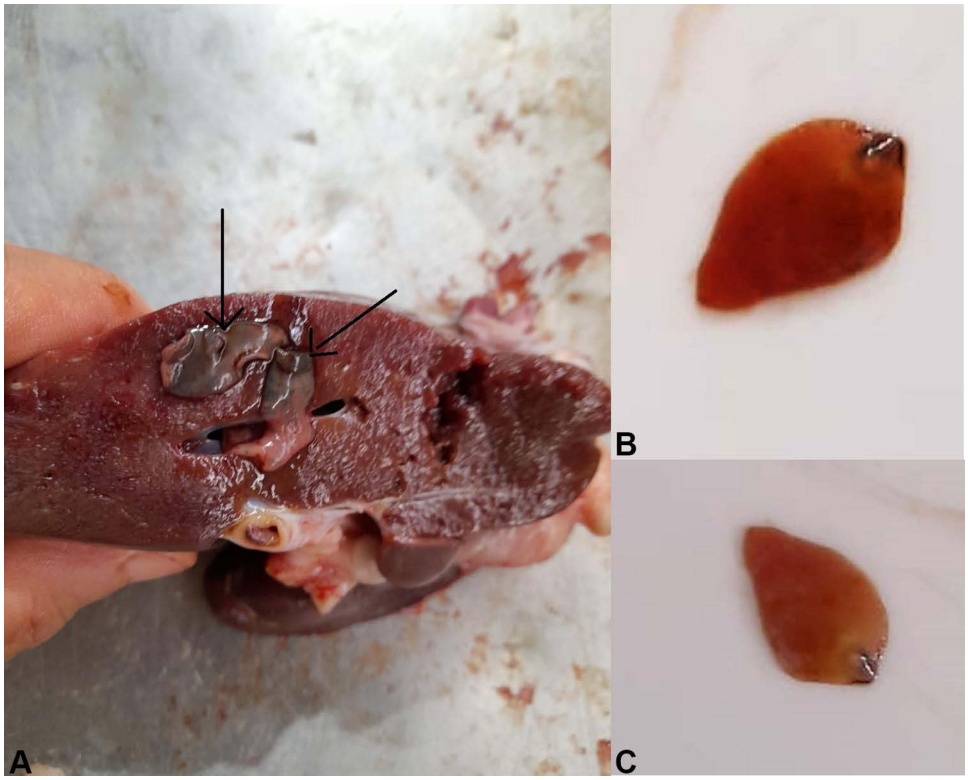

Figure 2 Showing *F. hepatica* adult worms isolated from infected cattle. (**A**): cattle liver infected with *F. hepatica* adult worms. (**B**) and (**C**) *F. hepatica* adult worms extracted from the infected cattle.

among male animals (6.9% in males vs 4.0% in females). However, the reported difference was not statistically significant (P=0.9). Similarly, higher infection rates were demonstrated in the adult animal group in comparison to young animals with no statistical significance (P=0.4) (Table 2).

Fasciola sp. adult worms (n=48) were collected from the infected cows (n=18). DNA was isolated and successfully amplified from all worms resulting in PCR products of the right size (493 bp) (Figure 3).

Next, we sequenced all PCR products (n = 48) and obtained 12 different DNA sequences. Frequency of sequences is shown in Table 3.

The variance of the obtained sequences ranged from 0.1887 to 1.0185. Detailed results of nucleotide variation are shown in Table 4.

Alignment of the sequences obtained from the current study identified the isolated worms as *F. hepatica* based on >97% similarity with *F. hepatica* sequences deposited in the GenBank database. A BLAST search showed that the

Table 2 Prevalence of *F. hepatica* in Cows at Jeddah Municipal Abattoir

Total Number		Number Examined N=325		Infected N=18		*P* value
		No.	(%)	No.	(%)	
Sex	Male	175	53.8	12	6.8	0.9
	Female	150	46.2	6	4.0	
Age	Young	185	56.9	5	2.7	0.4
	Adult	140	43.1	13	9.2	

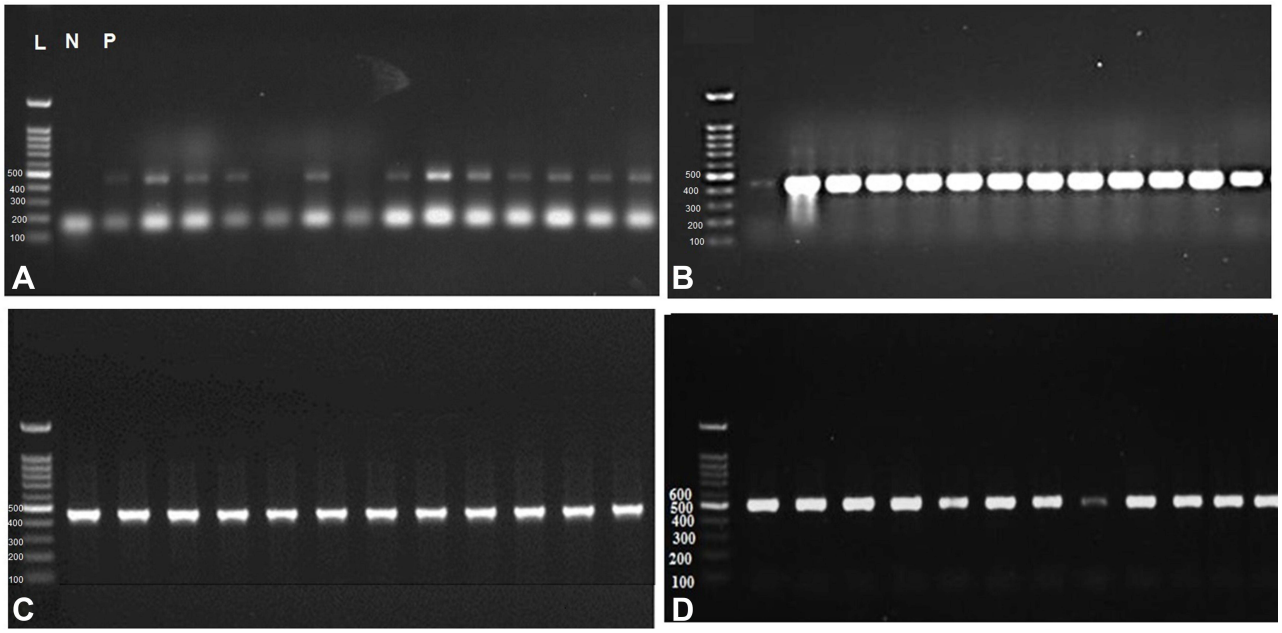

Figure 3 PCR products obtained from 48 *F. hepatica* adult worms isolated from cattle (**A**) Agarose gel electrophoretic analysis of PCR products of *F. hepatica* adult worms; L: 100 bp. Ladder, P: Positive control. N: negative control. The remaining lanes represent PCR products of *F. hepatica* adult worms at the size of 493 bp. (**B**), (**C**) and (**D**) showing the PCR products of *Fasciola* adult worms at 493 bp.

sequences are similar to *F. hepatica* isolated from cattle in Tanzania (JX236645 and EU282862), Eastern Europe (HM487172), Netherlands (FJ936028) and Iran (KY246450 and KY246451).

To construct the phylogenetic tree, the Maximum Likelihood method and Tamura-3 model were used. All sequences in the present study were compared with other sequences of *F. hepatica* deposited in the GenBank using *P. westermani* as an outgroup (Figure 4). Our isolates clustered with other *F. hepatica* sequences from the GenBank database which supported

Table 3 Showing the Frequency of 12 Sequences Obtained from 48 Adult Worms Isolated from 18 Infected Cows

Sequence Number	Frequency	Cattle Number
Sequence 1	5	1,3,8
Sequence 2	10	2,5,17,18
Sequence 3	7	5,6,9,11
Sequence 4	6	4,12,14,
Sequence 5	4	1,3,15,16
Sequence 6	2	15,17
Sequence 7	6	14,16,17
Sequence 8	3	7,9
Sequence 9	2	1,4
Sequence 10	1	5
Sequence 11	1	8
Sequence 12	1	10

Table 4 Genetic p-Distances Among *F. hepatica* Isolates in the Present Study Based on Sequence Analysis of Partial COI Gene

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12
H1												
H2	0.7007											
H3	0.7507	0.7890										
H4	0.7901	0.6051	0.7917									
H5	0.6537	0.5335	0.6924	0.4513								
H6	0.8159	0.7626	0.7831	0.8042	0.6698							
H7	1.0438	0.9098	0.9216	1.0182	0.9388	0.9656						
H8	0.8219	0.7959	0.7509	0.7512	0.7969	0.8032	0.5878					
H9	0.8748	0.8072	0.7901	0.8313	0.8358	0.8684	0.5887	0.3749				
H10	0.8020	0.7989	0.7559	0.8297	0.7700	0.4640	0.8823	0.8208	0.7955			
H11	0.2455	0.2126	0.2948	0.2049	0.1887	0.1659	0.4145	0.5500	0.5701	0.2105		
H12	0.8052	0.8351	0.7243	0.7571	0.6998	0.3475	1.0185	1.0098	1.0799	0.4427	0.0880	

BLAST search results and confirmed that all of our isolates were *F. hepatica*. Moreover, isolates from the present study were clustered in one group close to isolates from North Africa, South Africa, Turkey and Italy.

Discussion

Bovine fascioliasis is often presented as a subclinical disease that decreases animal productivity and results in significant economic loss. Several studies have demonstrated that the control of bovine fascioliasis is complicated by the complexity of the parasite's life cycle and emerging drug resistance.[24] Therefore, understanding the parasite life cycle, and evaluation of its genetic diversity and population structure would allow the development of effective control measures.[15,17,24]

In the present study, we aimed to identify the prevalence and phylogenetic analysis of fascioliasis in infected cattle for the first time in Jeddah city, Saudi Arabia. A total of 325 slaughtered cattle were examined. Eighteen cows (5.5%) were found to be infected with adult *F. hepatica* worms. Previous reports from several Saudi Arabian localities showed a wide range of infection rate. In Riyadh, Magda and Al-Megrin reported a higher infection rate in 2005 (21.9%) and Degheidy et al in 2012 (16.9%).[8,10] Mgzoub and Kasim, on the other hand, reported a lower infection rate in sheep from different regions in Saudi Arabia.[25] The authors reported an infection rate ranged from 0.18% to 2.4%.[25] Variable findings from different localities may be explained by the difference in sample size, environmental conditions, and the application of control measures.

To the best of our knowledge, this is the first study in Saudi Arabia to genetically characterize *Fasciola sp*. in cattle based on COI gene. Based on DNA sequence analysis, the isolated *Fasciola* adult worms in the present study were identified as *F. hepatica*. This finding is consistent with previous reports from Saudi Arabia.[26] Alajmi, reported *F. hepatica* as the predominant *Fasciola* species isolated from sheep in Riyadh, Saudi Arabia. The author identified both *F. hepatica* and *F. gigantica* in examined sheep, with *F. hepatica* inhabiting 80% of examined animals.[26] On the other hand, Shalaby et al reported equal infection rates for both *F. hepatica* and *F. gigantica* in imported sheep in Al Taif region in Saudi Arabia had.[27] Similar results have been reported by Farjallah et al.[28] The authors confirmed that all samples from Tunisia and Algeria samples belong to a single species, namely *F. hepatica*.[28] Based on both nuclear and mitochondrial sequences, reports from different worldwide locations had shown that *F. hepatica* has been identified as the most common *Fasciola* spp. in temperate regions while *F. gigantica* is the commonest in tropical countries of Africa.[20,29–32]

Sequence analysis of the PCR products of COI gene obtained from 48 *F. hepatica* worms isolated from 18 infected cows in the present study identified 12 different isolates. The phylogenetic analysis of the obtained isolates using MEGA X software

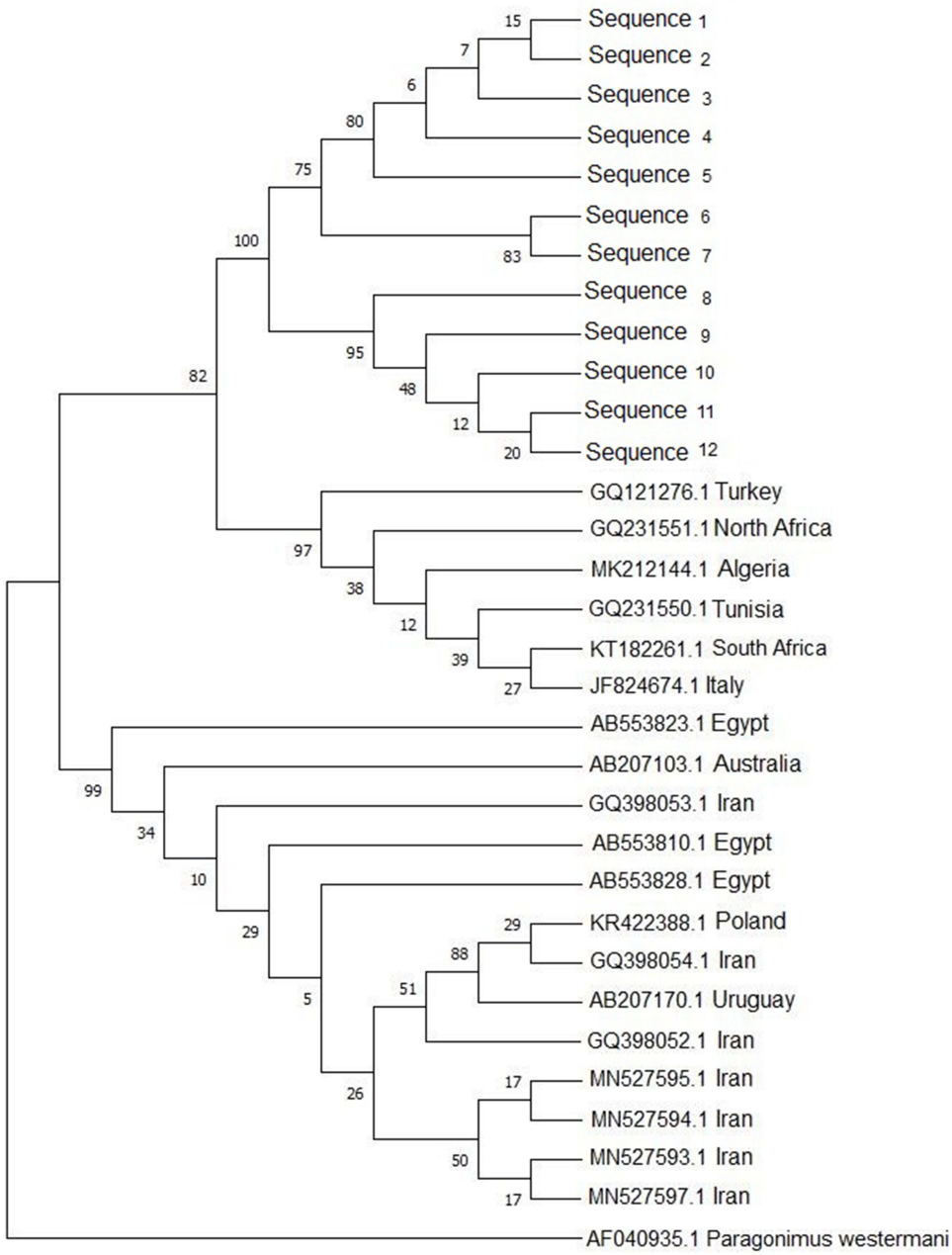

Figure 4 Phylogenetic relationship of *F. hepatica* isolated from cattle in Saudi Arabia computed by MEGA X the Maximum Likelihood (ML) and Tamura 3 parameter model from the partial (COI) gene nucleotide sequences, using *P. westermani* as an out-group strain. Numbers at the nodes indicate percentage of bootstrap support obtained in 1000 replicates.

showed a rooted tree with *Paragonimus westermani* as an outgroup. The resulting phylogenetic tree showed a close relationship of our isolates with those of other countries. The obtained isolates (12) clustered in one subclade and were closely related to isolates from Italy (JF824674.1),[33] Turkey (GQ121276.1),[14] South Africa (KT182261.1),[34] Algeria (MK212144.1),[35] North Africa (GQ231551.1),[28] Tunisia (GQ231550.1)[28].

Conclusion

Although there have been multiple publications on fascioliasis in Saudi Arabia, all of these reports are based on the morphology of eggs and adult worms, with limited molecular species identification. In the current study, we used COI as a molecular marker for genetic characterization of *Fasciola* adult worm isolated from infected cattle. Our findings

showed that *F. hepatica* was the only *Fasciola* species parasitizing cattle in Jeddah city, Saudi Arabia. The present study provided data for future monitoring of fascioliasis and identifying risk factors. Further studies using new molecular markers will be useful in the control and prevention of fascioliasis.

Study Limitation

Future research with a larger number of *Fasciola* adult worms from various regions throughout Saudi Arabia in order to figure out prevalence, risk factors, geographical distribution, how it spread, where it is thought to have originated, to other parts of the world, including Saudi Arabia.

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Disclosure

The authors declare that they have no conflicts of interest.

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