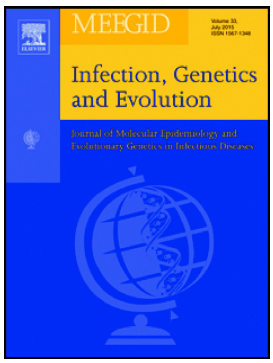

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Multigene typing and phylogenetic analysis of *Fasciola* from endemic foci in Iran

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Abstract

Fasciolosis is a public health problem originally transmitted from livestock. Although molecular analysis of nuclear and mitochondrial genes allow discrimination between the two known species of *Fasciola*, *F. hepatica* and *F. gigantica*, these markers do not permit the detection of hybrid forms. On the other hand, molecular analysis of the *pepck* and *pold* genes from *Fasciola* do permit the detection of hybrid isolates and this study has not yet been performed on specimens from human and domesticated animals in Iran. Therefore, the aim of this study was to molecularly analyze *Fasciola* isolates using both conventional and the two new genetic markers mentioned above. Fifty-three adult *Fasciola* worms were isolated from the livers of 28 domesticated animals, including sheep, cattle and goat. Moreover, an adult worm was obtained from the bile duct of an infected woman during endoscopic retrograde cholangio-

pancratography (ERCP). Targeted fragments from the ITS-1, NDI and COX1 genes were amplified using specific primers. PCR products were sequenced and genetically analyzed using MEGA v.7 and dnaSP software. Additionally, *pepck* and *pold* regions were amplified and analyzed using multiplex PCR and RLFP-PCR, respectively. Multiple alignment of sequenced fragments showed highest similarity among the ITS-1 sequences isolated from all four hosts in comparison to the other genes. Furthermore, sequence diversity across the COX1 was higher than the NDI and ITS-1. Indeed, diversity among sequences isolated from cattle was higher than those from sheep and goat in all three genes. Tajima's D and Fu's Fs and were negative and statistically significant for all the genes except ITS-1. Phylogenetic trees showed that the human *F. hepatica* isolate was closer to sheep isolates. The results of the *pepck* and *pold* analyses showed that all isolates were *F. hepatica* and there were no hybrid forms among samples. The molecular analyses corroborated this finding.

Keywords: *Fasciola hepatica*; Molecular analysis; *pold*; *pepck*; Human; Livestock.

1. Introduction

Fasciola hepatica, as a zoonotic helminth parasite, has a history as long as animal husbandry. Apart from human infections, *F. hepatica* is mainly known to be responsible for economic losses in livestock industry, worldwide (Beesley et al., 2018; Kaplan, 2001; Mas-Coma et al., 2009; Mehmood et al., 2017; Webb and Cabada, 2018). However, fasciolosis is a chronic infection of the bile ducts in both humans and animals and transmitted via contaminated food and water (Mas-Coma et al., 2005). According to the WHO report, during the recent years the reports of the infections have significantly increased and there is no *Fasciola*-free continent (WHO, 2019). However, based on global estimation, at least 2.7 million people are affected with this fluke (WHO, 2019). Thus, apart from its veterinary impacts, it seems that human fasciolosis is also a major public health concern, particularly in regions with traditional animal husbandry

(Ashrafi et al., 2014; Mas-Coma et al., 2014a; Mas-Coma et al., 2009, 2014b). Due to its clinical and epidemiological evidence, the burden of disease and other characteristics, the WHO has included fasciolosis within the group of foodborne trematode infections which are in the list of neglected tropical diseases (NTDs) (WHO, 2019).

F. hepatica and *F. gigantica* are two major species that reproduce bisexually through fertilization and have been separated from each other based on morphological criteria (Terasaki et al., 2001). Morphological hybrid forms have been reported from Japan and other Asian countries (Ashrafi et al., 2006; Itagaki et al., 2005a; Itagaki et al., 2005b; Itagaki et al., 2009; Moazeni et al., 2012; Moghaddam et al., 2004; Periago et al., 2008), which thought to be offspring of interspecies fertilization and probably reproduce parthenogenetically (Terasaki et al., 2001; Terasaki et al., 1998; Terasaki et al., 2000). Although molecular analyses of the nuclear ribosomal genes such as the internal transcribed spacer (ITS) 1, and the mitochondrial cytochrome oxidase (COX) 1 and the nicotiamide adenine dinucleotide dehydrogenase subunit I (NDI) genes can discriminate two major species of *Fasciola*, these markers are not able to characterize hybrid forms. To overcome this limitation, phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) genes were suggested as promising markers (Shoriki et al., 2016).

On the other hand, molecular studies in Iran have focused on the analysis of ITS-1 and 2, COX1 and ND1 (Bozorgomid et al., 2016; Galavani et al., 2016; Reaghi et al., 2016; Rouhani et al., 2017; Sarkari et al., 2017; Shokouhi and Abdi, 2018) and there is no report of molecular analysis of the hybrid forms using abovementioned new markers. Therefore, the current study aimed to perform molecular analyses on *Fasciola* isolates from human and animals using two new (*pepck* and *pold*) genetic markers together with the conventional genetic markers.

2. Materials and Methods

2.1.Parasite collection

2.1.1. Animal samples

A total of twenty-eight *Fasciola*-infected livers from domesticated animals including sheep (11), cattle (15) and goat (2) isolated from some parts of Ilam, Lorestan and Khuzestan provinces were included in this study (Fig 1). Accordingly, 53 adult worms (two flukes from each liver; with except the mono-helminth infected livers) were recovered from the examined livers.

2.1.2. Human sample

An adult worm was obtained from the bile ducts of an infected woman during endoscopic retrograde cholangio-pancratography (ERCP) in a hospital in Ilam. The infected patient was from a nomad society who grazes flock of sheep in West and North Lorestan to East and North-east of Ilam.

2.2.DNA extraction and amplification

DNA extraction was performed for each fluke using tissue DNA extraction kit (YektaTajhiz Azma [YTA], Tehran, Iran) according to manufacturer's instruction. Purified DNA was stored at -20ºC until further analyses. Afterwards, discriminative fragments of the ITS-1, COX1, NDI genes were amplified using the primers described elsewhere (Itagaki et al., 2005a). PCR reactions were carried out in final volume 25 µL containing 12.5 µL of 2X PCR ready-to-use mastermix with 1.5 mM $MgCl_2$ (Ampliqon, Denmark), 10 ρM of each primer and 2 µL from the template based on the standard cycling conditions: 95 ºC for 4 min, followed by 35 cycles at 94 ºC for 90 sec, 55 ºC for 90 sec and 72 ºC for 2 min with a final extension at 72 ºC for 5 min. PCR products were electrophoresed using 1.5% agarose gel, stained with 0.5 mg/mL of ethidium bromide and visualized by UV translluminator (Cleaver scientific Ltd, Warwickshire, United

Kingdom). Finally, PCR products were purified and sequenced using ABI 3130 sequencer. Generated sequences were edited and trimmed using Chromas v2.6.6 and BioEdit v7.0.5.3 software and submitted to the GenBank database with accession numbers: MK468803-MK468856 for NDI, MK447938-MK447991 for COX1 and MK377098-MK377151 for ITS-1.

2.3.Multiplex PCR and RFLP-PCR for the *pepck* and *pold* genes

To amplify the *pepck* fragment, multiplex PCR using the primers Fh-*pepck*-F, Fg-*pepck*-F, and Fcmn-*pepck*-R was performed as mentioned elsewhere (Shoriki et al., 2016). For the *pold* region, the fragment was amplified with the primers Fasciola-*pold*-F1 and R1 (Shoriki et al., 2016) and then digested by the restriction enzyme AluI (Roche, Germany). The resulted pattern was finally distinguished on 1.8% agarose gel.

2.4.Phylogenetic analysis

Multi alignment was performed for each gene using ClustalW incorporated in molecular and evolutionary genetic analysis (MEGA) v7 software (Kumar et al., 2016). In order to construct the phylogenetic trees, Maximum-likelihood together with Tamura 3-parameter were employed. In addition, to analyze the similarity of our sequences (for the NDI and COX1 genes) to previously reported isolates from other countries, Maximum-likelihood together with Tamura 3-parameter were employed. To assess the statistically strength of topology of the trees, bootstrap with 1000 replications was used.

2.5.Genetic diversity, neutrality tests

Moreover, DnaSP v5 was applied to assess the similarity between the specimen isolated from human with those obtained from domesticated animals. For this purpose, the number of haplotypes (Hn), haplotype diversity (Hd), segregating sites (S), nucleotide diversity (π), average number of pairwise nucleotide differences (K), as well as the neutrality indices including Fu's Fs

statistics and Tajima's D were calculated between and among the populations (Rozas et al., 2003).

3. Results

3.1.Molecular analysis of *pepck* and *pold*

The results of multiplex PCR of the *pepck* fragment showed that all 54 *Fasciola* isolates were *F. hepatica* and no hybrid form was detected among the samples. This result was confirmed by RFLP-PCR pattern of the *pold* gene.

3.2.Diversity indices and neutrality tests

Expected PCR fragments corresponding for the ITS-1, COX1 and NDI genes were amplified for all 54 animal and human isolates (Table 1). After trimming the sequences, fragment lengths 413-bp, 502-bp and 625 bp for COX1, NDI and ITS-1, respectively, were analyzed. Indeed, sequences NA15, NA16, NA20, NA29, NA36, and NA36 from the NDI genes together with sequence CO30 from the COX gene were excluded from the analyses due to their short length. Multiple alignment of sequenced fragments showed highest similarity among the ITS-1 sequences isolated from all four hosts in comparison to the other two genes. Accordingly, only 2 haplotypes (Ha) were retrieved from the ITS-1 gene while only one polymorphic (so-called: segregating [S]) site was seen throughout the sequenced fragment of the ITS-1 gene. Furthermore, sequence diversity through the COX1 fragment was higher than that in the NDI and ITS-1 fragments (Table 2).

Molecular analysis also showed that Hd and π in the ITS-1 fragment were 0.037 and 0.00006, respectively that were lower than that in the NDI and COX1 fragments. Furthermore, the NDI fragment represented higher Hn, Hd, S and K indices, demonstrating a higher sequence diversity of this fragment among the *F. hepatica* isolates (Table 2 and Fig 2).

Neutrality tests showed negative values consisted of Tajima's D and Fu's Fs that were statistically significant for all genes except ITS-1. However, the negative values for Tajima's D and Fu's Fs suggested excess of rare alleles/sweep gene and recent population expansion (Rozas et al., 2017), respectively (Table 2).

Molecular analyses based on the hosts showed higher diversity among sequences isolated from cattle than those from sheep and goat in all three genes. Apart from the COX1 gene among sheep sequences, the highest number of haplotypes and Hd were seen among the NDI and ITS-1 genes isolated from cattle (Table 3).

3.3.Phylogenetic analysis

Phylogenetic analyses using ML trees showed high similarity among the ITS-1 sequences, while diversity among the COX1 and NDI genes was high. In addition, our findings showed that there was no classification based on the origin of hosts, while ML trees revealed that the human *F. hepatica* isolate was more similar to sheep isolates than those from goad and cattle (Fig 3). Furthermore, Bayesian trees showed that there was a major haplotype for both the COX1 and NDI genes, while there was no correlation between hosts (Fig 4).

4. Discussion

The analysis of *pold* and *pepck* genes showed that all *Fascioa* isolates from both animals and human were *F. hepatica.* Although presence of *F. gigantica* and hybrid forms using morphological criteria (Aryaeipour et al., 2017; Ashrafi et al., 2006; Ashrafi et al., 2015; Yakhchali et al., 2015) and conventional molecular markers (Bozorgomid et al., 2016; Imani Baran et al., 2017; Reaghi et al., 2016) was reported, this is the first study that molecularly investigated the occurrence of this form in Iran using new genetic markers, *pold* and *pepck*. Interestingly, the results of molecular analysis of *pold* and *pepck* showed that there was no

intermediate form among isolates from west of Iran while hybrid forms were previously reported by Ashrafi et al. (2015), from north of the country.

Here for the first time in Iran, molecular analysis of *F. hepatica* from human and comparison to *Fasciola* isolates from animal hosts (cattle, sheep and goat) in same foci were performed. Fasciolosis, as a major public health problem in tropical countries, has been frequently reported from grazing animals in Iran (Arjmand et al., 2014; Aryaeipour et al., 2014; Aryaeipour et al., 2017; Askari et al., 2018; Bozorgomid et al., 2016, Imani Baran et al., 2017). In Iran, up to now, several known small to large human outbreaks were described from the western and northern provinces, respectively (Ashrafi, 2015). As one of the largest human outbreaks of *F. hepatica* in the world, it was estimated that more than ten thousand peoples were infected by the parasite in Bandar-Anzali and Rasht districts, Guilan province, north of Iran in 1989 (Massoud, 1989).

During the years, although the annual incidence of human fasciolosis has reduced, new foci were described. It seems that western provinces of Iran are the most important new foci for human fasciolosis (Hatami et al., 2012; Mowlavi et al., 2010; Sarkari et al., 2012). However, the human outbreaks reported from these regions were smaller than that previously described from north of Iran. Based on the serological studies, the presence of anti-*Fasciola* antibody among evaluated populations was reported lower than 2% (Asadian et al., 2013; Sarkari et al., 2012). Nonetheless, concerning this fact that traditional animal husbandry is a common job in the west regions of Iran, this region should be considered as high risk foci for the human fasciolosis outbreaks.

Molecular analyses of *Fasciola* in Iran have a long history and many studies were conducted on the molecular characterization of this parasite among livestock (Bozorgomid et al.,

2016; Galavani et al., 2016; Moazeni et al., 2012; Reaghi et al., 2016; Rokni et al., 2010; Tadayon et al., 2015). However, there is only one study that characterized *F. hepatica* from human origins. Accordingly, Sharifiyazdi et al. (2012), molecularly analyzed two *F. hepatica* isolates from north of Iran with 100% similarity in the ITS-1 and ND1 genes to *F. hepatica* isolated from a Japanese man. As a limitation, in abovementioned study the human cases were not molecularly compared with animal *Fasciola* isolates from the studied region; therefore, they did not describe the probable origins of the human infections. Interestingly, in the current study we compared the human *Fasciola* case with *F. hepatica* isolated from livestock in same regions suggesting sheep as the potential source of infection.

In the current study, we used ribosomal (ITS 1) and mitochondrial (NDI and COX1) markers for characterization and analysis of *F. hepatica* isolates from both human and animal cases. As expected, the lowest diversity was observed among the ITS-1 sequences (Bozorgomid et al., 2016). However, this gene was suggested as a useful marker for species discrimination; therefore, based on the morphological and ITS-1 analyses, all studied isolates were identified *F. hepatica.* Notably, this result was confirmed by the findings of *pold* and *pepck* analyses that showed there was no hybrid forms among the samples. However, two haplotypes were determined in cattle samples using the ITS-1 fragment, while only one haplotype was seen among samples obtained from sheep and goat.

Interestingly, the findings of phylogenetic analyses using ML algorithms on the NDI and COX1 showed that isolated *F. hepatica* from human was molecularly close to sheep isolates. It was previously suggested that the fast mutation rate of the mitochondrial genes like COX1 and NDI, makes them useful markers for differentiation of molecularly close isolates (Bozorgomid et al., 2016). Therefore, it seems that ML algorithms on the NDI and COX1 genes may reveal

potential origin of human infections. Notably, the infected patient was from a nomad society who grazed flock in studied regions. On the other hand, the previous studies in west of Iran demonstrated the presence of *F. hepatica* in this region (Hatami et al., 2012; Shafiei et al., 2014; Shokouhi and Abdi, 2018) and since traditional animal husbandry like free-grazing flock is a common job, it seems that the risk of human infection with *F. hepatica* in west of Iran is high.

To compare molecular diversity among our isolates with those reported from other countries, phylogenetic trees using Maximum likelihood were constructed. As a result, 31 COX1 haplotypes were detected among 68 sequences including one major haplotype consisted of 24 sequences and several minor haplotypes. However, the results showed that 44 NDI haplotypes were detected among 74 analyzed sequences. Regarding to the results, there was one major NDI haplotype consisted of 19 sequences and several minor NDI haplotypes among the *Fasciola* isolates. These results suggest that *F. hepatica* isolates from Iran were relatively variable populations. However, the high diversity across the NDI and COX1 genes in *F. hepatica* isolates might be resulted from its broad host range, as well as the presence of the infection for a long time in a region. It was supported by the recent study conducted by Askari *et al.* (2018), who reported *F. hepatica* eggs in paleofaeces of a donkey from ancient Iran (224-651AD). Therefore, Iran is thought to be near by original locality of *F. hepatica*, because the populations were more variable than other countries like Egypt populations (Amer et al., 2011).

5. Conclusion

The current findings showed that all *Fasciola* isolates from domesticated animals and human case were *F. hepatica*. Indeed, the results of the analysis of the *pold* and *pepck* genes demonstrated that there were no hybrid forms among our *Fasciola* isolates. Phylogenetic and molecular analyses represented the lowest molecular diversity across the ITS-1 fragment.

However, the COX1 and ND genes revealed high molecular diversity. Our molecular findings also suggested that *F. hepatica* isolates in Iran were variable populations proposing Iran as an original locality of *F. hepatica.*

Declarations

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Ethical approval

All procedures performed in this study were in accordance with the ethical standards (IR.SBMU.RIGLD.REC.1396.164) released by Ethical Review Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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Availability of data and materials

The data associated with this manuscript consisted of GenBank accession numbers are mentioned throughout the manuscript.

Author Contributions

Conceived and designed the experiments: HM. Performed the experiments: EJ YO. Analyzed the data: HM TI. Sample providing: AS KCh Contributed reagents/materials/analysis tools/positive samples: HAA MRZ. Wrote the paper: HM TI. All authors read and approved the final version of the manuscript.

Consent for publication

All authors of this manuscript declare that we have seen and approved the submitted version of this manuscript.

Informed consent

In the case of human fascioliasis, the patient agreed to publish demographic data in the manuscript.

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Figure Captions

Fig 1: The map of Iran shows provinces (in red) that the sampling was performed. Domesticated and human samples were collected from regions among the circle.

Fig 2: Pattern of mismatch distribution through the **A)** COX1, **B)** NDI, and **C)** ITS-1 genes. Exp (Expected) means the value of expected nucleotide diversity and Obs (Observed) means the value of nucleotide diversity that observed in these amplified fragments.

Fig 3: Phylogenetic analyses of *F. hepatica* sequences from **A)** COX1, and **B)** NDI genes, respectively. The trees were assembled based on the Maximum-likelihood test and the Tamura 3-parameter model using MEGA7 software. The percentage of bootstrap above the branches supports the interior branches. Branches without numbers have bootstrap values < 70%.

Fig 4: Phylogenetic analyses of *F. hepatica* sequences from **A)** COX1 and **B)** NDI genes, respectively. The trees were constructed based on Maximum-likelihood test and the Tamura 3-parameter model using MEGA7 software. The figures show the correlation between our *F. hepatica* isolates with *Fasciola* sequences reported from other countries.

Table 1: Accession numbers of *F. hepatica* isolated from cattle, sheep, goat and human based on the NDI, COX1 and ITS-1 genes.

No.	Hosts	NDI		COI		ITS-1	
		Acc. numbers	**Abbrev. name**	**Acc. numbers**	**Abbrev. name**	**Acc. numbers**	**Abbrev. name**
1	Cattle	MK468803	NA1	MK447938	CO1	MK377098	IT1
2	Cattle	MK468804	NA2	MK447939	CO2	MK377099	IT2
3	Cattle	MK468805	NA3	MK447940	CO3	MK377100	IT3
4	Cattle	MK468806	NA4	MK447941	CO4	MK377101	IT4
5	Sheep	MK468807	NA5	MK447942	CO5	MK377102	IT5
6	Sheep	MK468808	NA6	MK447943	CO6	MK377103	IT6
7	Sheep	MK468809	NA7	MK447944	CO7	MK377104	IT7
8	Sheep	MK468810	NA8	MK447945	CO8	MK377105	IT8
9	Cattle	MK468811	NA9	MK447946	CO9	MK377106	IT9
10	Cattle	MK468812	NA10	MK447947	CO10	MK377107	IT10
11	Cattle	MK468813	NA11	MK447948	CO11	MK377108	IT11
12	Cattle	MK468814	NA12	MK447949	CO12	MK377109	IT12
13	Cattle	MK468815	NA13	MK447950	CO13	MK377110	IT13
14	Cattle	MK468816	NA14	MK447951	CO14	MK377111	IT14
15	Sheep	MK468817	NA15	MK447952	CO15	MK377112	IT15
16	Sheep	MK468818	NA16	MK447953	CO16	MK377113	IT16
17	Sheep	MK468819	NA17	MK447954	CO17	MK377114	IT17
18	Sheep	MK468820	NA18	MK447955	CO18	MK377115	IT18
19	Cattle	MK468821	NA19	MK447956	CO19	MK377116	IT19
20	Cattle	MK468822	NA20	MK447957	CO20	MK377117	IT20
21	Cattle	MK468823	NA21	MK447958	CO21	MK377118	IT21
22	Sheep	MK468824	NA22	MK447959	CO22	MK377119	IT22
23	Sheep	MK468825	NA23	MK447960	CO23	MK377120	IT23
24	Cattle	MK468826	NA24	MK447961	CO24	MK377121	IT24
25	Cattle	MK468827	NA25	MK447962	CO25	MK377122	IT25
26	Sheep	MK468828	NA26	MK447963	CO26	MK377123	IT26
27	Sheep	MK468829	NA27	MK447964	CO27	MK377124	IT27
28	Cattle	MK468830	NA28	MK447965	CO28	MK377125	IT28

29	Cattle	MK468831	NA29	MK447966	CO29	MK377126	IT29
30	Cattle	MK468832	NA30	MK447967	CO30	MK377127	IT30
31	Cattle	MK468833	NA31	MK447968	CO31	MK377128	IT31
32	Sheep	MK468834	NA32	MK447969	CO32	MK377129	IT32
33	Sheep	MK468835	NA33	MK447970	CO33	MK377130	IT33
34	Goat	MK468836	NA34	MK447971	CO34	MK377131	IT34
35	Goat	MK468837	NA35	MK447972	CO35	MK377132	IT35
36	Cattle	MK468838	NA36	MK447973	CO36	MK377133	IT36
37	Cattle	MK468839	NA37	MK447974	CO37	MK377134	IT37
38	Sheep	MK468840	NA38	MK447975	CO38	MK377135	IT38
39	Sheep	MK468841	NA39	MK447976	CO39	MK377136	IT39
40	Sheep	MK468842	NA40	MK447977	CO40	MK377137	IT40
41	Sheep	MK468843	NA41	MK447978	CO41	MK377138	IT41
42	Sheep	MK468844	NA42	MK447979	CO42	MK377139	IT42
43	Sheep	MK468845	NA43	MK447980	CO43	MK377140	IT43
44	Cattle	MK468846	NA44	MK447981	CO44	MK377141	IT44
45	Cattle	MK468847	NA45	MK447982	CO45	MK377142	IT45
46	Cattle	MK468848	NA46	MK447983	CO46	MK377143	IT46
47	Cattle	MK468849	NA47	MK447984	CO47	MK377144	IT47
48	Cattle	MK468850	NA48	MK447985	CO48	MK377145	IT48
49	Cattle	MK468851	NA49	MK447986	CO49	MK377146	IT49
50	Cattle	MK468852	NA50	MK447987	CO50	MK377147	IT50
51	Cattle	MK468853	NA51	MK447988	CO51	MK377148	IT51
52	Goat	MK468854	NA52	MK447989	CO52	MK377149	IT52
53	Goat	MK468855	NA53	MK447990	CO53	MK377150	IT53
54	Human	MK468856	NA54	MK447991	CO54	MK377151	IT54

Table 2: Number of analyzed sequences, genetic diversity and neutrality indices based on the "COX1, ITS-1 and NDI" genes of *F. hepatica*. No: number of sequences; Hd: haplotype diversity; Hn: number of haplotypes; π: nucleotide diversity; S: segregating sites; k: average number of pairwise nucleotide diversity.

Analyzed genes	No	Hn	Hd	π	S	K	Neutrality indices	
							Tajima's D	Fu's Fs
ITS-1	54	2	0.037	0.00006	1	0.037	-1.09496†	-1.701†
NDI	48	20	0.829	0.01331	81	6.457	-2.44072**	-3.343#
COX1	53	19	0.786	0.00597	34	2.438	-2.35342**	-10.411***

Statistically significance, P < 0.02

† Statistical not significant, P > 0.10

Statistical significance: **, P < 0.01

Statistical significance: ***, P < 0.02

Table 3: Comparative analysis of the number of sequences and genetic diversity of the ITS-1, COX1 and NDI genes obtained from cattle, sheep and goat. No: number of sequences; Hd: haplotype diversity; Hn: number of haplotypes; π: nucleotide diversity; S: segregating sites; k: average number of pairwise nucleotide diversity.

Analyzed genes in each host		No	Hn	Hd	π	S	K
ITS-1	Cattle	29	2	0.06897	0.0001	1	0.06897
	Sheep	20	1	0	0	0	0
	Goat	4	1	0	0	0	0
COX1	Cattle	28	12	0.78042	0.00488	15	1.98942
	Sheep	20	11	0.89526	0.00740	24	3.02105
	Goat	4	3	0.83333	0.00613	5	2.5
NDI	Cattle	26	11	0.83077	0.01122	46	5.44308
	Sheep	18	8	0.75163	0.01132	37	5.49020
	Goat	3	3	1	0.04124	30	20

Author Contributions

Conceived and designed the experiments: HM. Performed the experiments: EJ YO. Analyzed the data: HM TI. Sample providing: AS KCh Contributed reagents/materials/analysis tools/positive samples: HAA MRZ. Wrote the paper: HM TI. All authors read and approved the final version of the manuscript.

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Thank you in advance for your consideration,

Sincerely,

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Highlights

1. *Fasciola* isolates were collected from sheep, cattle and goat and also from a human case.
2. Conventional genetic markers (COX1, ITS-1 and NDI), as well as *pepck* and *pold* were molecularly analyzed.
3. The results of *pepck* and *pold* showed that all isolates were *F. hepatica* and there were no hybrid forms.
4. The NDI gene showed higher genetic diversity than the COX1 and ITS-1 fragments
5. Sequence diversity among cattle isolates was higher than that in sheep and goat in all three genes.