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Original Article

Specific Detection of *Fasciola hepatica* and *F. gigantica* in Infected Domesticated Animals Using High-Resolution Melting Analysis (HRM)

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Abstract

Background: It is difficult to make an exact morphological distinction between *Fasciola hepatica* and *Fasciola gigantica*. We used High Resolution Melting analysis (HRM) method to differentiate the *F. hepatica* species from *F. gigantica* in order to differentiate them.

Methods: Overall, 80 adult liver flukes were collected from infected slaughtered animals including cattle, sheep and goats from Lorestan Province, western Iran from Sep 2015 to Aug 2017. Genomic DNA was extracted using commercial DNA extraction kit. The multilocus sequences of mDNA including COX1, COX3 and ND6 were amplified employing real-time PCR & HRM analysis. Specific and universal primer pairs were designed for differentiation *Fasciola* spp.

Results: Universal primers cannot be used to distinguish between these two species, but in the contrary, specific primer pairs of each species could differentiate them properly. Molecular identification using specific primer pairs were consistent.

Conclusion: HRM is a simple, fast and reliable method for detecting and differentiating *F. hepatica* from *F. gigantica* and can be used for diagnostic and epidemiological purposes.

Keywords: *Fasciola hepatica; Fasciola gigantica*; Fasciolosis; Polymerase chain reaction (PCR)

Introduction

Fascioliasis emerges as a parasitic disease of human and animal and considers as a neglected worldwide disease (1). Fascioliasis can be transmitted through contaminated foods and is among the most dangerous parasitic diseases (2). It has been observed an increase in human infection with fascioliasis for four decades that predicts the number of 2.400.000 people in 61 counties with populations 180 million at fascioliasis risk (3). Human and animal infections can occur by two species of fascioliasis including *Fasciola hepatica* and *F. gigantica*. The latter appears to be a more serious causative infection agent rather than the former. This can be due to the size of parasite in *F. gigantica* (4). Both species, however, show some overlaps across geographical areas (5).

It is of concern to distinguish between species of fascioliasis according to different environmental

and biological features these intermediate hosts can indicate. Moreover, hybridization may occur in the case of species co-existence. *F. hepatica* and *F. gigantica* intermediate hosts have been reported some countries of Asia including China (6), Iran (5), Korea (7), Japan (8), Vietnam (9, 10), and Africa such as Egypt (11, 12). In general, the distinction between both species is difficult according to the criteria of phenotypic (13).

Crucial findings may be revealed in the case of performing an investigation on the species in Iran, given the increase in the number of sporadic infections in some provinces this country including Gilan, Mazandaran, Tehran, Kermanshah, Kohgiluyeh and Boyer-Ahmad, Khuzestan, and Lorestan, respectively (14). Besides, regarding the importance of geography of Lorestan as well as its neighboring province including Kermanshah, it demands researches to address the diagnosis and genotype of *Fasciola*. Currently, identifying parasites requires molecular methods that address the progress, epidemiology, diagnosis, prevention, treatment, genetic pattern detection, drug, and vaccine resistance of this process. Regarding this, an accurate and reliable molecular method should be adopted that allows identification of the *Fasciola* species and a distinction between both (15, 16).

For utilizing a quick and accurate method for two species differentiation (i.e. *F. hepatica* and *F. gigantica*), the current paper implements the method of HRM. Sampling is performed using the slaughterhouses located in the province of Lorestan.

Materials and Methods

Sample collection

Eighty adult trematodes of *F. hepatica* and *F. gigantica* were located within the bile ducts of infected cattle (n=30 samples), goat (n=25 samples) and sheep (n=25 samples), were collected from slaughterhouses in Lorestan Province, West of Iran including Khorramabad, Aligudarz and Borujerd (Fig. 1) from Sep 2015 to Aug 2017. The flatworms were extensively washed in physiological saline and preserved in >70° (v/v) ethanol and then frozen at -20 °C until DNA extraction.

The study was conducted in the Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Ethics Committee of Tehran University of Medical Sciences approved the current study (IR.TUMS.SPH.REC.1398.203).

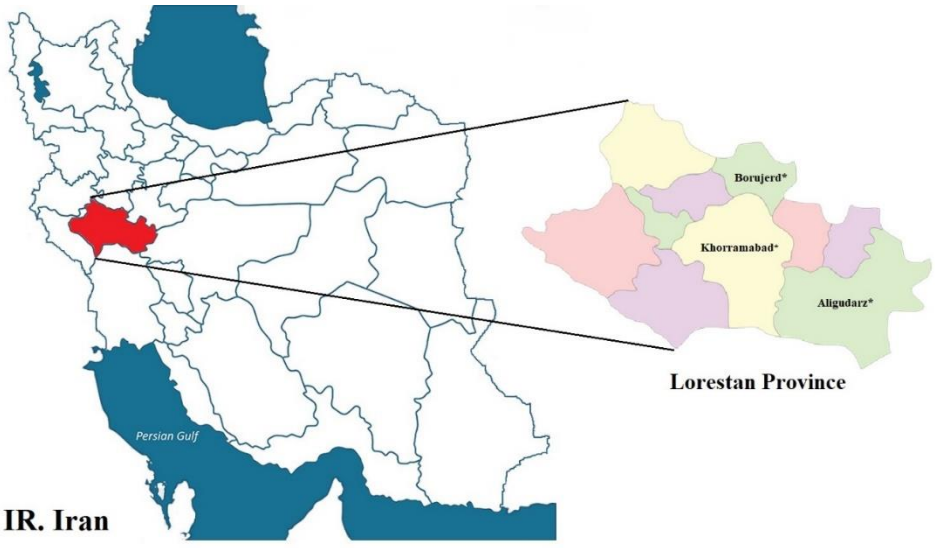

Fig. 1: Geographical location of Lorestan province. Sites of collected of cattle, goat and sheep in the districts under study (identified with name and black stars). These areas include: Khorramabad, Aligudarz and Borujerd City

DNA extraction

Before DNA extraction, all individual worms washed three times with PBS to remove the ethanol. Genomic DNA was extracted from small part of apical region of adult trematodes to avoid entry female genitalia that likely to outer sperm. DNA from individual worms was extracted using a DNA Extraction Kit (Bioneer, Korea), according to the manufacturer's reference protocols with some modifications. The concentration of the extracted DNA was evaluated by NanoDrop (Thermo Scientific, Rockford, IL, USA), and after that, the samples were stored at -20 °C for further analysis.

DNA amplification and HRM

For the amplification of cytochrome c oxidase subunit 1 (COX1) gene for specific detection of *F. hepatica,* (forward AHS-F.h-COX1-F primer [5-AGATTTGGGCTTTGGTTGCTCGG-3] and reverse AHS-F.h-COX1-R primer [5-GACAAACAAACACAAGCAGGCAAT-3] and, (forward AHS-F.g-COX1-F primer [5-GCTTTGAGTGCTTGGTTGTTGC-3] and reverse AHS-F.g-COX1-R primer [5-TATATGACGACCAGTACCCTCGC-3] for specific detection of *F. gigantica*. Moreover, the multilocus sequences were amplified using universal primers. For the ND6 gene, primers (forward AHS-ND6-F [5-TTCTATTCATATACCTAACC-3] and reverse AHS-ND6-R [5-TAACCACAACATTAACAC-3] as well as for amplification of COX3 gene, primers (forward AHS-COX3-F [5-GAAAGTTGTTAGGCTTGTG-3] and reverse AHS-COX3-R [5-AATCATCCTCAACCGTTAT-3] design was carried out using the sequences available on GenBank and in continuation, Primers were obtained using the software PRIMER BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and rechecked with Beacon Designer 8.12, PREMIER BIOSOFT software from the consensus sequence obtained by the multiple alignments, with an expected amplicon size of 440, 240, 221, 180 bp for COX1 *F. hepatica*, COX1 *F. gigantica*, ND6 and COX33 gene sequences respectively.

PCR was performed in 20 mL final reaction volume containing 4 mL master mix (HOT FIRE-Pol® EvaGreen® HRM Mix; Solis BioDyne, Tartu, Estonia), 10 mL distilled water, 0.5 mL of each primer, and 5 mL of template DNA. We defined three different temperature zones for specific annealing and best amplification for each primer and in continuation, the enzymatic reaction was performed under the following condition with specific temperature for each zone: the reaction mixture was heated for initial denaturation step at 95 °C for 10 min, followed by 30 and 40 cycles of amplification related to COX1 and COX3/ND6 respectively were performed at 95 °C for 30 sec for denaturation step, 63.5 °C, 52 °C and 48.5 °C for 30 sec belonging to COX1, COX3 and ND6 for annealing part, 72 °C for 30 sec related to extension portion, and a final extension step at 72 °C for 7 min after 30 and 40 cycles. High Resolution Melting temperature was raised from 65 °C to 85 °C and 95°C. During this process, the amplicons obtained from PCR were denatured prior to the development of melting curves in the inflexion point where changes in fluorescence with respect to changes in temperature (dF/dT) were recorded with a ramp of 0.3 °C/sec. Fluorescence dye signaling was measured after each cycle. The kit contained the novel double-stranded DNA-binding fluorescent dye, EvaGreen, and an optimized HRM PCR master mix buffer, consisting of HotStarTaq plus DNA polymerase, Q-Solution, and dNTPs.

Real-time PCR was carried out in a Mini Opticon real-time PCR detection system (Applied Biosystems Step One Plus Inc., CA, USA). The Real-Time amplification result and T_m analysis were obtained using the Step One Plus™ software ver. 2.3(Life technologies®). T_m analysis was repeated three times in each run to confirm the repeatability of the T_m assay by estimating the T_m variation within a PCR amplification (intra-assay), and between PCR amplification (inter-assay). The coefficient of variation (CV) was calculated by dividing the standard deviation (SD) by the arithmetic

mean of the measured values of Tm (CV=[SD]/mean value).
Furthermore, to check the uniformity of temperature in the cycler block, a number of samples were re-amplified at different positions of the cycler block during the same amplification cycle. The intra-assay CVs represent the mean CVs of the results obtained from the replications of all the *Fasciola* spp. in the same run. The inter-assay CVs represent the mean CVs for the results obtained from 3 separate runs for each gene (Table 1).

Table 1: Mean T_m, SD, and CV calculated based on intra- and inter-assay of each gene sequences (COX1 *F. hepatica*, COX1 *F. gigantica*, ND6 and cox3) of *Fasciola.* spp.

Gene	*Mean Tm (°C)*	*SD*	*Intra-assay CV* (%)*	*Inter-assay CV (%)*
COX1 *F. hepatica*				
F. hepatica	84.6°C	0.13	0.03	0.11
F. gigantica	---	---	---	---
COX1 *F. gigantica*				
F. hepatica	---	---	---	---
F. gigantica	80.2°C	0.11	0.09	0.16
ND6				
F. hepatica	78.26°C	0.11	0.08	0.12
F. gigantica	76.64°C	0.13	0.06	0.10
COX3				
F. hepatica	80.77°C	0.10	0.05	0.12
F. gigantica	80.87°C	0.11	0.04	0.11

Results

According to real-time PCR coupled with HRMA assay, using four sequence gene, among eighty samples of *F. hepatica* and *F. gigantica* including 30 cattle, 25 goats and 25 sheep, were amplified using partial sequences of COX1, ND6 and COX3 gene of *Fasciola* spp., and then HRM was performed. Tm analysis was repeated three times in each run to confirm the repeatability of the T_m assay described in Table 1. Overall, 61 and 19 Isolates were identified as *F. hepatica* and *F. gigantica* respectively. All pair of primers related to four different sequences gene were amplified excellently and confirmed *Fasciola* spp. and among them, the primers pair designed to amplify COX1 gene were able to distinguish *F. hepatica* from *F. gigantica*. Thus, the primer pair designed to COX1 gene to detection of *F. hepatica* was not capable of amplified *F. gigantica* and on the contrary the primer pair was designed to amplified COX1 gene of *F. gigantica* unable to amplify the genome of *F. hepatica*. Insilico analysis of ND6 and COX3, we have seen that primers designed for these two genes, like COX1 primers, can be able to amplify specifically the *Fasciola* spp. genome, but in experimental analysis the results showed that primers pair designed for the ND6 and COX3 genes were not capable of specific amplification of *F. hepatica* and *F. gigantica* genome and could not separate them from each other. For this reason, we named these primers pair, universal primers, because they were able to amplify both genomes of *F. hepatica* and *F. gigantica*.
The real-time PCR melting curve and HRMA results indicated that the mean Tm, SD, CV calculated based on intra- and inter-assay of the COX1, ND6 and COX3 were separated by each

strain of *Fasciola* spp. respectively. Real-time PCR amplification and HRMA of the *Fasciola* spp. detection by multilocus sequencing are shown in Figs. 2-7 according to four different sequences genome.

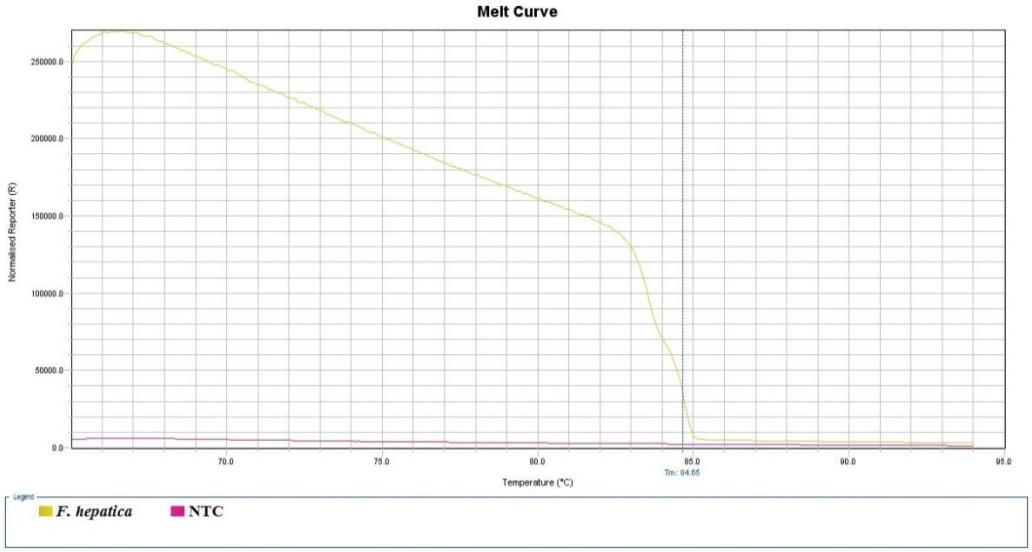

Fig. 2: HRM based on (EvaGreen) Aligned Melt curves analyses and identified *F. hepatica* in cattle, goat and sheep using specific primer pair of COXI gene

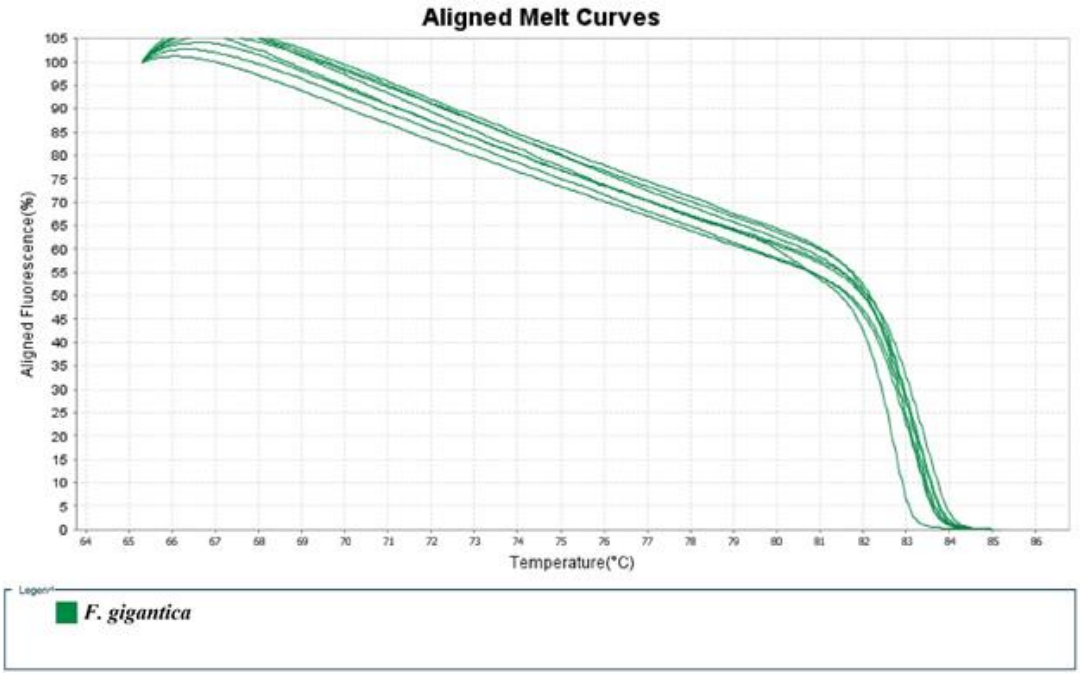

Fig. 3: HRM based on (EvaGreen) Aligned Melt curves analyses and identified *F. gigantica* in cattle, goat and sheep using specific primer pair of COXI gene

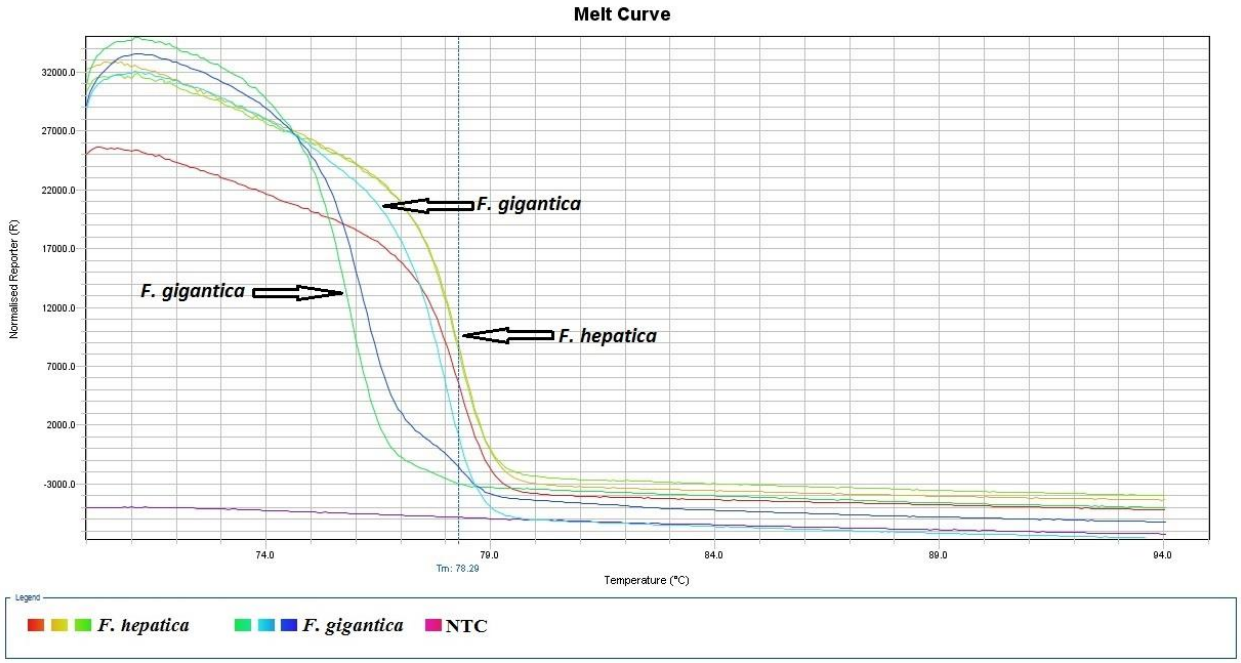

Fig. 4: HRM based on (EvaGreen) Aligned Melt curves analyses and identified *F. hepatica* and *F. gigantica* in cattle, goat and sheep using specific primer pair of ND6 gene

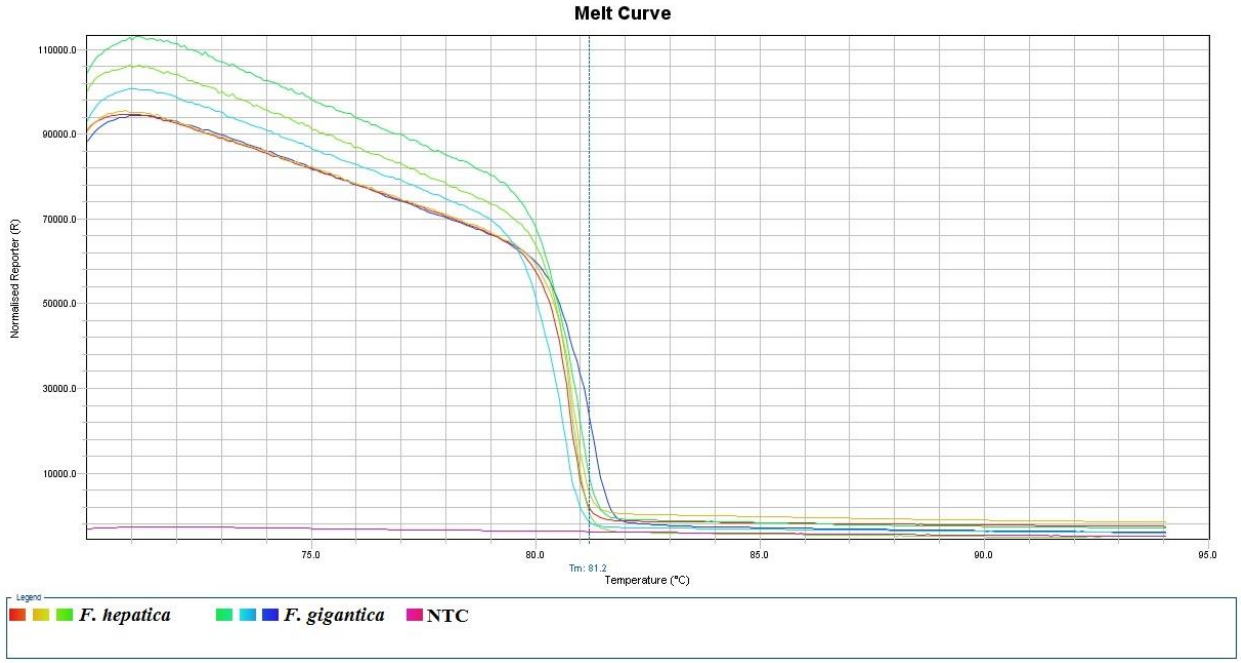

Fig. 5: HRM based on (EvaGreen) Aligned Melt curves analyses and identified *F. hepatica* and *F. gigantica* in cattle, goat and sheep using specific primer pair of COX3 gene

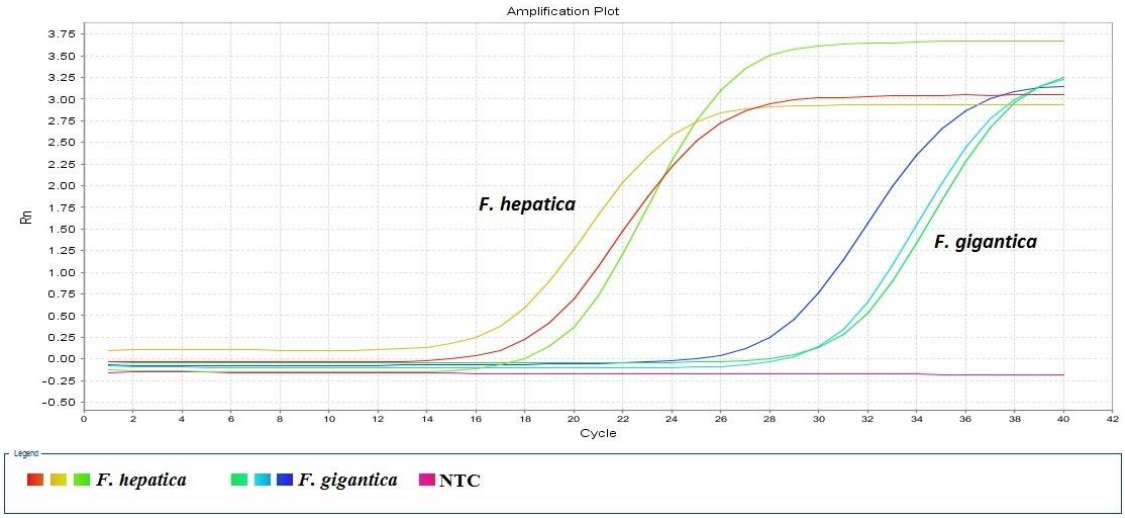

Fig. 6: Amplification plot of *F. hepatica* and *F. gigantica* in cattle, goat and sheep using specific primer pair of COX3 gene

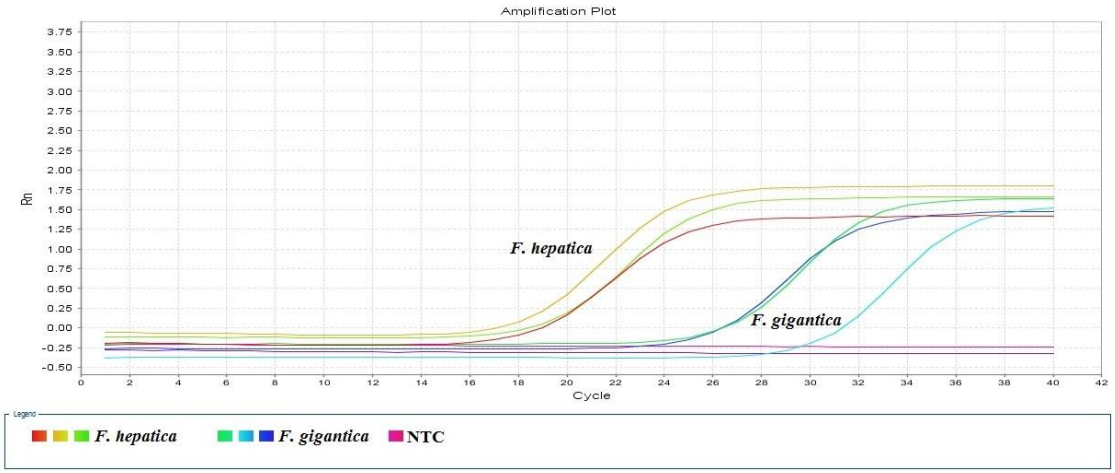

Fig. 7: Amplification plot of *F. hepatica* and *F. gigantica* in cattle, goat and sheep using specific primer pair of ND6 gene

Discussion

In this study, we investigated different gene sequences to separate *F. hepatica* from *F. gigantica*. Overall, 61 samples from 80 collected samples were related to *F. hepatica* and 19 samples to *F. gigantica*. In the present study, three mitochondrial genes including Cox1, Cox3, and ND6 were used to evaluate multilocus genome .The primers of these genes were designed so that *F. hepatica* or *F. gigantica* could be specifically amplified, of which only the Cox1 gene could specifically be amplified and separated *F. hepatica* from *F. gigantica*, but on the contrary other two genes unlike Insilico's evaluations, could not specific amplification of genome and simultaneously it had amplified the *F. hepatica* and *F. gigantica* genomes, which were unable to distinguish these two species due to the existence of overlap in their T_m.

So far, many studies have been done in the world and in Iran to separate *F. hepatica* from *F. gigantica* that using of different genres such as ITS1, ITS2, COX1 and ND1 and different methods such as PCR, RFLP-PCR and Real-time PCR could sepa-

rate *F. hepatica* and *F. gigantica* (7, 17-19). The present study is the first multilocus genomes study using HRM technique to identity different species of *Fasciola* and specific amplification of each species using specific primers. In the current study, a certain amount of the adult worm cervical area of both species was removed for extraction of DNA, and all samples were extracted using the same method. The C_t of all *F. hepatica* samples was lower than *F. gigantica* as the mean of Ct are 19.9 and 28 respectively, easily separated in the amplification plot using COX3 and ND6 genes (Figs. 6 and 7).

So far, several studies have been done using HRM technique in Iran (20, 21) and other parts of the world. A study was done in Iran to evaluate the genetic diversity of *Echinococcus granulosus* using the COX1 gen (21).

In Egypt, using the ITS1 and COX1 genes, all the *Fasciola* were *Hepatica* species (22). Comparison of *F. hepatica* with *F. gigantica* indicated that *F. hepatica* differs more than 6 nucleotides with *F. gigantica* from Egypt, Japan, and Indonesia, whereas only one nucleotide of interspecific difference was observed in the COX1 gene (22). In Nepal, 81 *Fasciola* isolated was investigated using gene areas of ITS1 and ND1, and 75.3% *F. hepatica* and 24.7% *F. gigantica* were identified (23). In Iran, in Kohgiluyeh and Boyer-Ahmad Province, 70.7% and 29.3% of the samples were identified regarding to *F. hepatica* and *F. gigantica*, respectively (24). In another study in Iran, 96% *F. hepatica* and 4% *F. gigantica* were detected in the samples (25).

Conclusion

F. hepatica and *F. gigantica* are both common in Iran, therefore, an appropriate method for differentiating these two species is necessary. HRM is a simple, fast and reliable method for detecting and differentiating *F. hepatica* from *F. gigantica* and can be used for diagnostic and epidemiological purposes. The HRM method can be used for the proper identification of *Fasciola* species.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

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Conflict of interests

The authors declare that there is no conflict of interests.

References

1. Hotez PJ, Savioli L, Fenwick A (2012). Neglected tropical diseases of the Middle East and North Africa: review of their prevalence, distribution, and opportunities for control. *PLoS Negl Trop Dis,* 6:e1475.
2. Mahami-Oskouei M, Dalimi A, Forouzandeh-Moghadam M, Rokni M (2011). Molecular identification and differentiation of *Fasciola* isolates using PCR-RFLP method based on internal transcribed spacer (ITS1, 5.8 S rDNA, ITS2). *Iran J Parasitol,* 6(3):35-42.
3. Rim H-J, Farag H, Sornmani S, Cross J (1994). *Food-borne trematodes: ignored or emerging?* Elsevier Current Trends.
4. Valero MA, Bargues MD, Khoubbane M, et al (2016). Higher physiopathogenicity by *Fasciola gigantica* than by the genetically close *F. hepatica*: experimental long-term follow-up of biochemical markers. *Trans R Soc Trop Med Hyg,* 110 (1):55-66.
5. Amor N, Halajian A, Farjallah S, Merella P, Said K, Slimane BB (2011). Molecular characterization of *Fasciola* spp. from the endemic area of northern Iran based on nuclear ribosomal DNA sequences. *Exp Parasitol,* 128(3):196-204.
6. Peng M, Ichinomiya M, Ohtori M, Ichikawa M, Shibahara T, Itagaki T (2009). Molecular characterization of *Fasciola hepatica*, *Fasciola*

gigantica, and aspermic *Fasciola* sp. in China based on nuclear and mitochondrial DNA. *Parasitol Res,* 105(3):809-15.

7. Agatsuma T, Arakawa Y, Iwagami M, Honzako Y, Cahyaningsih U, Kang S-Y, Hong S-J (2000). Molecular evidence of natural hybridization between *Fasciola hepatica* and *F. gigantica*. *Parasitol Int,* 49(3):231-238.
8. Itagaki T, Kikawa M, Sakaguchi K, Shimo J, Terasaki K, Shibahara T, Fukuda K (2005). Genetic characterization of parthenogenic *Fasciola* sp. in Japan on the basis of the sequences of ribosomal and mitochondrial DNA. *Parasitology,* 131:679-685.
9. Itagaki T, Sakaguchi K, Terasaki K, Sasaki O, Yoshihara S, Van Dung T (2009). Occurrence of spermic diploid and aspermic triploid forms of *Fasciola* in Vietnam and their molecular characterization based on nuclear and mitochondrial DNA. *Parasitol Int,* 58(1):81-85.
10. Le TH, Van De N, Agatsuma T, Nguyen TGT, Nguyen QD, McManus DP, Blair D (2008). Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. *Int J Parasitol.,* 38(6):725-730.
11. Amer S, Dar Y, Ichikawa M, Fukuda Y, Tada C, Itagaki T, Nakai Y (2011). Identification of *Fasciola* species isolated from Egypt based on sequence analysis of genomic (ITS1 and ITS2) and mitochondrial (NDI and COI) gene markers. *Parasitol Int,* 60(1):5-12.
12. Periago M, Valero M, El Sayed M, et al (2008). First phenotypic description of *Fasciola hepatica*/ *Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infect Genet Evol,* 8(1):51-58.
13. MAS CS, Bargues M (1997). Human liver flukes: a review. *Res Rev Parasitol*, 57(3-4):145–218.
14. Ashrafi K (2004). A survey on human and animal fascioliasis and genotypic and phenotypic characteristics of fasciolids and their relationship with lymnaeid snails in Gilan province, northern Iran [Ph. D thesis]. *School of Public Health, Tehran University of Medical Sciences, Iran.*
15. Esteban JG, Bargues MD, Mas-Coma S (1998). Geographical distribution, diagnosis and treatment of human fascioliasis: a review. *Res Rev Parasitol*, 58 (I): 13-42.
16. Marcilla A, Bargues M, Mas-Coma S (2002). A PCR-RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica*. *Mol Cell Probes,* 16(5):327-333.
17. Hosseini-Safa A, Rokni MB, Mosawi SH, Heydarian P, Azizi H, Davari A, Aryaiepour M (2019). High-Resolution Melting Analysis as an Appropriate Method to Differentiate between *Fasciola hepatica* and *F. gigantica*. *Iran J Public Health,* 48(3):501-507.
18. Rokni MB, Mirhendi H, Mizani A, Mohebali M, Sharbatkhori M, Kia EB, Abdoli H, Izadi S (2010). Identification and differentiation of *Fasciola hepatica* and *Fasciola gigantica* using a simple PCR-restriction enzyme method. *Exp Parasitol,* 124(2):209-213.
19. Saki J, Khademvatan S, Yousefi E (2011). Molecular identification of animal *Fasciola* isolates in southwest of Iran. *Australian Journal of Basic and Applied Sciences,* 5(11):1878-1883.
20. Hosseini-Safa A, Mohebali M, Hajjaran H, Akhoundi B, Zarei Z, Arzamani K, Davari A (2018). High resolution melting analysis as an accurate method for identifying *Leishmania infantum* in canine serum samples. *J Vector Borne Dis,* 55(4):315-320.
21. Pestechian N, Safa AH, Tajedini M, et al (2014). Genetic diversity of *Echinococcus granulosus* in center of Iran. *Korean J Parasitol,* 52(4):413-18.
22. Omar MA, Metwally AM, Sultan K (2013). Molecular and phylogenetic status of *Fasciola* sp., of cattle in Qena, Upper Egypt. *Pak J Biol Sci, PJBS,* 16(15):726-730.
23. Shoriki T, Ichikawa-Seki M, Devkota B, Rana HB, Devkota SP, Humagain SK, Itagaki T (2014). Molecular phylogenetic identification of *Fasciola* flukes in Nepal. *Parasitol Int,* 63(6):758-762.
24. Shafiei R, Sarkari B, Sadjjadi SM, Mowlavi GR, Moshfe A (2014). Molecular and morphological characterization of *Fasciola* spp. isolated from different host species in a newly emerging focus of human fascioliasis in Iran. *Vet Med Int,* 2014:405740.
25. Bozorgomid A, Nazari N, Rahimi H, et al (2016). Molecular characterization of animal *Fasciola* spp. isolates from Kermanshah, Western Iran. *Iran J Public Health,* 45(10):1315-21.