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Short communication

DETECTION OF *FASCIOLA HEPATICA* USING NESTED-PCR IN THE SLAUGHTERHOUSES OF ALBORZ, IRAN

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Summary

Rezaee Kalvani, A., L. Abdolmohammadi Khiav & S. R. Hosseini, 2023. Detection of *Fasciola hepatica* using nested-PCR in the slaughterhouses of Alborz, Iran. *Bulg. J. Vet. Med.* (online first).

Fasciola hepatica is a parasitic liver trematode that causes fasciolosis in humans and dairy animals. Traditional detection of infection is based on a microscopic examination with low sensitivity. Therefore, this study was carried out to develop an accurate and rapid method to detect *F. hepatica* in Alborz province. In this cross-sectional study, 386 samples were collected from livers of cattle and sheep in three slaughterhouses of Alborz. DNA was extracted, and nested-PCR was carried out based on the *F. hepatica* ITS-2 sequence. DNA amplification showed first and second PCR products with expected size of 336 and 208 bp respectively, as unique for *F. hepatica*. The results showed no cross-reaction with the negative control. Thirty-five liver samples were positive for this parasitic infection. Totally, the prevalence of *F. hepatica* in the slaughterhouses of Alborz, Iran was 9.07%. This is the first study of the molecular detection of *F. hepatica* using nested-PCR in Alborz. It is a sensitive and rapid method that will help evaluate the prevalence rate of *F. hepatica* infection.

Key words: cattle, detection, epidemiology, *Fasciola hepatica,* nested-PCR, sheep

F. hepatica (sheep liver fluke) is a parasitic liver trematode that causes fasciolosis. This parasite infects dairy animals, especially sheep and cattle, resulting in mortality, decreased lactation, and fertility among the dairy animals' population (Mignaqui *et al.*, 2020). *F. hepatica* also causes foodborne infection, a zoonotic disease with clinical signs including fever, a swollen liver, and abdominal pain (Amiri *et al.*, 2021) that has affected millions people throughout the world from Latin America, Europe, the Caribbean, Africa, the Middle East, Oceania, and Asia (Karagulle *et al.*, 2022). The life cycle of *F. hepatica* comprises egg, miracidium, cercaria, metacercaria, and adult fluke. Snails are an intermediate host that can transmit the infection to humans or livestock (Lu *et al.*, 2018). Animal fasciolosis causes mortality and morbidity in dairy animals. In the UK *F. hepatica* has

caused an annual severe economic loss of £40.4 million in the cattle industry in 2005 (Amiri *et al.*, 2021). In Iran, animal fasciolosis has a long history and has always been considered a crucial veterinary issue. The first human case of the disease was diagnosed in Iran in 1955. Since then, a few cases of the disease have been reported yearly in different provinces. The first significant outbreak of human fasciolosis occurred in Gilan in 1989, and more than 10,000 people were infected in Bandar-Anzali and Rasht. The second outbreak of human fasciolosis occurred ten years later and 5000 people were infected (Ashrafi, 2015). The Mazandaran province is another prevalent region of fasciolosis in Iran too (Moghaddam *et al*., 2004) with reported prevalence of animal fasciolosis from 27.3% to 75%. Cases of human fasciolosis were also reported from other provinces, including Ardabil, Khuzestan, Kohgyluye va Buyerahmad, Kermanshah Province, and Yasuj and Boyer-Ahmad (Ashrafi, 2015).

Due to the fact that fasciolosis is a significant health problem, especially in Iran (Torgerson *et al*., 2015) and economic losses in dairy animals, the detection of fasciolosis is critical. Parasitological methods are the golden standard methods, but are unable to diagnose all cases of fasciolosis accurately due to low sensitivity (Brockwell *et al.*, 2013). Fas2-enzyme-linked immunosorbent assay (Fas2-ELISA), immunofluorescence assay (IFA), and indirect haemagglutination assay (IHA) are used for the detection of disease. Serological tests have disadvantages, similarly to other tests. PCR-based methods have been developed for detecting fasciolosis: whole-genome sequencing (WGS) and single nucleotide polymorphism (SNP), random amplified polymorphic DNA (RAPD), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Hamoo *et al*., 2019), quantitative real-time polymerase chain reaction (qPCR) assay (Calvani *et al.,* 2017) and nested-PCR (Alizadeh *et al*., 2022). In recent years, molecular markers including internal transcribed spacer 1 (ITS1), ITS2, 28S rRNA, *COXI*, and *NADI*, which provided valuable information for epidemiological studies have been developed. In this study, a nested-PCR assay for the identification of *F. hepatica* in cattle and sheep was developed using ITS-2 – a specific sequence without cross-reaction that results in higher sensitivity that amplifies the cytochrome C oxidase gene (Martínez-Pérez *et al*., 2012). For the detection of *F. hepatica* in cattle and sheep faecal samples by molecular-based methods, optimisation of the method is necessary due to inhibitory substances in faecal samples such as bile, proteases, and nucleases (Repetto *et al*., 2013). So, the selection of a DNA extraction method is a very critical step that influences the efficacy of molecular-based methods. Molecular-based methods also require equipment such as a thermocycler, but they are helpful with high specificity and sensitivity that can detect cases of fasciolosis correctly (Alizadeh *et al*., 2022). There is a little information about fasciolosis, especially in this geographical localisation of Iran. So, the aim of this study was the development of a rapid and accurate method to detect *F. hepatica* in the slaughterhouses of Alborz, Iran.

A cross-sectional study was performed on 386 samples collected from livers of cattle (n=80) and sheep (n=306) in three slaughterhouses in Karaj, Alborz, Iran. Liver samples were immediately transported to the laboratory under aseptic

conditions. All samples were stored at −20 °C until used.

Briefly, 20 mg liver tissue was digested by proteinase K (50 mg/mL) at 56 °C overnight, and then DNA was extracted using a Genomic DNA Kit (TIANGEN, China). The quantity of DNA was evaluated through 260 nm absorbance using the NanoDrop spectrophotometer for all samples. Then the quality of the DNA was evaluated by electrophoresis (1% agarose gel).

Specific primers that were designed from ITS-2 sequences of *F. hepatica* (accession number: AB553729.1) are listed in Table 1. For the first reaction, Fh-F and Fh-R were used as primers. The final volume of PCR was 25 µL including 12.5 µL 2× Premix Taq™, 2 µL primers (0.5 µM), 1 µL template DNA (100 ng/µL), and 9.5 µL double-distilled water. The nested-PCR conditions were as follows: 95 °C for 5 min, 35 cycles of 30 s at 95 °C, 30 s at 56 °C, and 40 s at 72 °C, and a final extension at 72 °C for 7 min. For the second reaction, n-F and n-R were used for primers, and the PCR product of the first reaction was substituted as a template DNA. The annealing temperature for this reaction was 30 s at 57 °C. In the first reaction, the genomic DNA of *F. hepatica* was used as a positive control. *M. orientalis* and no-template DNA was used as negative control. The PCR product of the positive and negative controls was applied in the second reaction (Huang *et al*., 2019). Then, 10 µL PCR products were mixed with 2 µL 6× gel loading dye. Gel electrophoresis was done using 3% agarose gel, which was then stained with ethidium bromide (0.5 µg/mL) for 15 minutes and visualised by UV-light transilluminator (GelDoc, BioRad, USA).

The information about liver infection with *F. hepatica* is given in Table 2. Based on the results, the rate of *F. hepatica* was higher in males than females in Alborz province. The rate of infected livers was mainly related to sheep aged over two years. DNA amplification showed an expected size of 336 and 208 bp at the first and second of product PCR, respectively, as unique for *F. hepatica*. No PCR product was amplified with the genomic DNA of *M. orientalis*. The results showed that there was no cross-

Table 1. Primers, sequence and product sizes of the PCR (Huang *et al*., 2019).

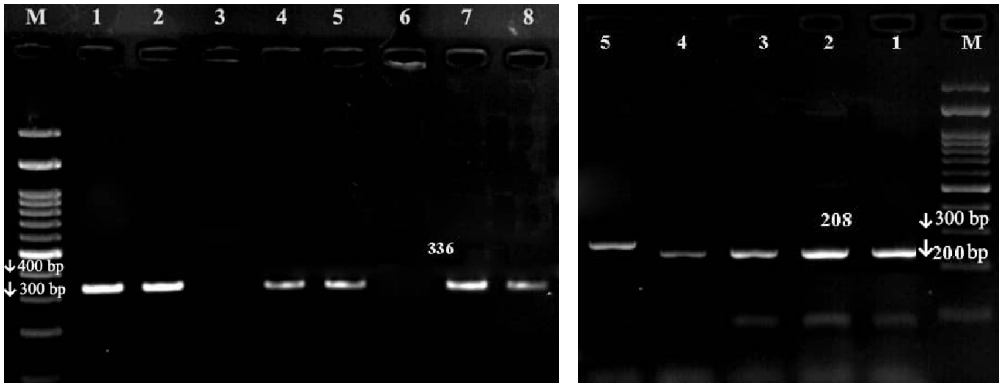
Primers	Sequence (5′-3′)	Product size (bp)
Fh-F	ATATTGCGGCCATGGGTTAG	336
Fh-R	CCAATGACAAAGTGACAGCG	
n-F	TATCACGACGCCCAAAAAGTC	208
n-R	GATCGCCAAACACACTGACA	

Table 2. The information of liver infection with *F. hepatica* based on gender, age and clinical signs of the animal

Clinical signs	Gender		Age (years)
	Male	Female	
Liver with dark spots	5	1	<1
Inflamed liver with dark spots	9	2	1–2
Livers with rounded edges	14	4	> 2

Table 3. Frequency of livers infection with *F. hepatica* in three slaughterhouses based on host

Slaughterhouse	Cattle (positive/ total number)	Sheep (positive/ total number)	Positive percentag (slaughterhouse)
I	0/20	12/65	14.11
II	0/0	11/126	8.73
III	0/60	12/115	6.85
Total	0/80	35/306	9.07



A **B**

Fig. 1. A) PCR amplification with the first primers (Fh-F and Fh-R). Lane M: 100 bp DNA marker. Lane 1: samples. Lane 2: *F. hepatica* positive control; lane 3: rumen fluke *M. orientalis* (negative control); lane 4–8: samples. **B)** PCR amplification with the second primers (n-F and n-R). Lane M: 100 bp DNA marker; lane 1: *F. hepatica* positive control; lanes 2–5: samples.

reaction. Thirty-five liver samples were positive for *F. hepatica* (Fig. 1).

The occurrence of livers infected with *F. hepatica* in three slaughterhouses based on the host was given in Table 3. Based on the results, all cases of *F. hepatica* infection were in sheep. Totally, the prevalence of *F. hepatica* in the slaughterhouses of Alborz province was 9.07%.

F. hepatica causes fasciolosis in dairy animals resulting in mortality and morbidity. Detection of this agent is helpful for the control of disease. Traditional detection is based on a microscopic examination with low sensitivity. So, this study was carried out to develop of an accurate and rapid technique to detect *F. hepatica.* The outbreaks of human fasciolosis were reported previously in Iran (Ashrafi, 2015). WHO listed Iran as a country with problems with fasciolosis (Torgerson *et al*., 2015), but there is still no regular programme for disease prevention which necessitates rapid and accurate detection of *F. hepatica*. Detection methods are not very suitable, so, the choice of diagnostic method is crucial. Parasitological methods are the golden standard methods, although these tests lack high sensitivity. Serological tests are suitable for the early stages of infection but may not be suitable for current infection because of circulating antibodies for several months after treatment (Salimi-Bejestani *et al*., 2005). The molecular-based techniques will be helpful with this regard and are the most

promising for the diagnosis of infection compared with traditional methods, including microscopic examination and ELISA (Amiri *et al*., 2021). In this study, we developed the nested-PCR assay for the identification of *F. hepatica* in cattle and sheep using ITS-2 and determination of the prevalence rate of *F. hepatica* infection in Karaj, Alborz province. A nested-PCR was developed to detect *F. hepatica* in snails as an intermediate host in northwestern China (Huang *et al*., 2019). In another study, nested-PCR was used as a diagnostic tool for human fasciolosis in Tehran, Iran and based on results, infected samples with *F. hepatica* were detected with high accuracy (Aryaeipour *et al.,* 2020). Alizadeh *et al*. (2022) also used semi-nested-PCR as a diagnostic tool for *F. hepatica* and compared this method with indirect ELISA. Based on the report, high agreement and similarity were observed between indirect ELISA and semi-nested PCR. Saki *et al*. (2021) also reported 100% similarity between nested PCR and ELISA in the detection of *Toxoplasma gondii*. Another study reported that most negative faecal culture samples were positive using nested PCR (Moghaddassani *et al*., 2011). *T. gondii and Histomonas meleagridis* have also reported high sensitivity of nested PCR, even compared with real-time PCR (Aryaeipour *et al.,* 2020), which showed the value of this assay for diagnosis of disease cases. In this study, no PCR product was amplified with the genomic DNA of *M. orientalis*. The results showed no cross-reaction similarly to other studies (Huang *et al*., 2019; Alizadeh *et al*., 2022). Based on our results, all cases of infection with *F. hepatica* were in sheep.

Totally, the prevalence of *F. hepatica* from the slaughterhouses of Alborz, Iran was 9.07%. Eslami *et al.* (2009) reported that the prevalence rate of fasciolosis for sheep in Gilan, Iran was 32%. However, in another study from Gilan, all cases of fasciolosis were reported only in cattle (Hosseini *et al*., 2010). Mahami-Oskouei *et al.* (2011), found out that 1.2% of sheep were infected with *F. hepatica*. The same result was reported in the study of Hosseini *et al.* (2012). In another study, the prevalence rate was 20% for sheep livers in Ardabil, Iran (Khanjari *et al*., 2010). Movassagh-Ghazani *et al.* (2008), reported that 8.57% of sheep in Eastern Azerbaijan were infected with *F. hepatica*, a rate similar to that reported by Khosravi & Babaahmady (2012) and the present study. According to the information above, the prevalence rate of fasciolosis is high in most studies, especially in the northern Iran. Due to the economic loss of fasciolosis, it is recommended to carry out nested-PCR to identify of fasciolosis, and good food hygiene, including washing vegetables, cooking food properly, and treating infected animals using albendazole and triclabendazole.

CONCLUSION

This is the first study of nested-PCR detection for *F. hepatica* in Alborz. Nested-PCR is a specific and sensitive method for the detection of *F. hepatica* without cross-reaction with *M. orientalis*, which is proposed for the detection of infection cases.

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