

Original Article

Detection of *Fasciola Hepatica* in Lori Sheep Using Polymerase Chain Reaction and Conventional Diagnostic Methods in Western Iran

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Abstract

Fascioliasis is an emerging and important food and water-borne disease in human communities which has become one of the most important health challenges in countries, like Iran. It causes weight loss, a decrease in feed conversion ratio as well as milk and meat production, and also reduces fertility in animals the prevalence of fasciolosis is increasing in some regions of the world due to various factors. Different methods have been used for the detection of *Fasciola hepatica* in animals. This study is the first to detect *F. hepatica* in Lori sheep using polymerase chain reaction (PCR) and conventional diagnostic methods in Western Iran. During three months, 195 fecal samples were collected from sheep in Lorestan province, Iran, using the stratified random sampling method. The conventional diagnostic methods, including wet mount microscopic examination and concentration assays, as well as the PCR technique targeting the intergenic spacer gene of *F. hepatica*, were used for the detection of the parasite in sheep. In total, 4 (2.1%) out of 195 examined stool samples were positive for *F. hepatica* based on the conventional assays. The PCR test was positive for *F. hepatica* in 7 (3.6%) samples of 195 studied specimens. Statistical analyses of the data revealed that there is a significant difference between the results of diagnostic methods for *F. hepatica* detection ($P=0.0421$). Finally, the results showed that PCR has more diagnostic sensitivity, compared to conventional diagnostic methods, including the concentration techniques and microscopic examination. Hence, it can be advised to use PCR for the detection of *F. hepatica* in sheep.

Keywords: Conventional diagnostic methods, Fascioliasis, Polymerase Chain Reaction

Détection de *Fasciola Hepatica* chez les Moutons Lori à L'aide de la Réaction en Chaîne par Polymérase et des Méthodes de Diagnostic Conventionnelles dans L'ouest de l'Iran

Résumé: La fasciolose est une maladie émergente et importante d'origine alimentaire et hydrique dans les communautés humaines qui est devenue l'un des défis sanitaires les plus importants dans des pays comme l'Iran. Elle entraîne une perte de poids, une diminution du taux de conversion alimentaire ainsi que la production de lait et de viande, et réduit également la fertilité chez les animaux. La prévalence de la fasciolose augmente dans certaines régions du monde en raison de divers facteurs. Différentes méthodes ont été utilisées pour la détection de *Fasciola hepatica* chez les animaux. Cette étude est la première à détecter *F. hepatica* chez les moutons Lori en utilisant la réaction en chaîne par polymérase (PCR) et des méthodes de diagnostic conventionnelles dans l'ouest de l'Iran. Pendant trois mois, 195 échantillons fécaux ont été collectés sur des moutons dans la province

du Lorestan, en Iran, en utilisant la méthode d'échantillonnage aléatoire stratifié. Les méthodes de diagnostic conventionnelles, y compris l'examen microscopique humide et les dosages de concentration, ainsi que la technique de PCR ciblant le gène d'espacement intergénique de *F. hepatica*, ont été utilisées pour la détection du parasite chez les moutons. Au total, 4 (2.1%) des 195 échantillons de selles examinés étaient positifs pour *F. hepatica* sur la base des tests conventionnels. Le test PCR a été positif pour *F. hepatica* dans 7 (3.6%) échantillons sur 195 échantillons étudiés. Les analyses statistiques des données ont révélé qu'il existe une différence significative entre les résultats des méthodes de diagnostic pour la détection de *F. hepatica* ($P=0.0421$). Enfin, les résultats ont montré que la PCR a une plus grande sensibilité diagnostique, par rapport aux méthodes de diagnostic conventionnelles, y compris les techniques de concentration et l'examen microscopique. Par conséquent, il peut être conseillé d'utiliser la PCR pour la détection de *F. hepatica* chez les moutons.

Mots-clés: Méthodes de diagnostic conventionnelles, Fasciolose, Réaction en chaîne par polymérase

1. Introduction

Fasciola hepatica, *Fasciola gigantica*, and their hybrids cause fasciolosis which is an infectious disease and has huge global importance (Torgerson and Macpherson, 2011). It is estimated that fasciolosis causes the loss of two billion dollars annually (Calvani et al., 2018). It leads to weight loss, a decrease in feed conversion ratio as well as milk and meat production, and also reduces fertility in animals (Mahami-Oskouei et al., 2011).

Fascioliasis is an important emerging food and water-borne disease in human communities as well and has become one of the most important health challenges in some countries like Iran (Ghavami et al., 2009). Economic losses caused by fascioliasis in Iranian animal farming are estimated to be thousands of dollars annually (Imani-Baran et al., 2012). The prevalence of fasciolosis is increasing in some regions of the world due to various factors, including climate variations and environmental changes made by humans (Rojo-Vazquez et al., 2012; Martinez-Valladares et al., 2013).

Early and accurate detection of *Fasciola* species helps to control fascioliasis effectively (Saki et al., 2011). Conventional methods are extensively used for the diagnosis of fascioliasis. Its diagnosis relies on the microscopic observation of *Fasciola* eggs in the fecal samples of infected animals (Shahzad et al., 2012). However, the conventional methods are unable to

diagnose fascioliasis accurately, recognize intraspecific differences of *Fasciola* species, or identify eggs of different trematode (Robles-Perez et al., 2013).

Polymerase Chain Reaction (PCR) has recently become a notable technique for this purpose. Real-time PCR is a common method in this field and can detect parts of genomic DNA (Alamian et al., 2019). Several studies have been carried out to investigate the genetic characteristics of adult forms of *Fasciola* species isolated from slaughtered animals in different regions of Iran. However, their results have not been compared with those of the studies conducted using conventional methods (Rokni et al., 2010; Shahbazi et al., 2011; Yakhchali et al., 2015).

A lot of people in Lorestan province, West of Iran work as stockbreeders, and they mostly breed sheep. It was necessary to perform a study on the diagnosis of *F. hepatica* in Lori sheep by PCR. Therefore, this study was conducted for the first time to investigate and compare the efficiency of PCR and conventional microscopic examination in the detection of *F. hepatica* in Lori sheep.

2. Material and Methods

2.1. Sample Collection

In total, 195 fecal samples were collected from sheep in Lorestan province by stratified random sampling procedure during three months. The samples were collected from 11 cities in this province, including

Khorramabad, Aligoudarz, Poledokhtar, Delfan, Kouhdasht, Selseleh, Azna, Boroujerd, Chegeni, Doroud, Roomeshkan, as well as their districts, and their villages. Cluster sampling was used for sample collection in different urban and rural areas.

Distribution of samples/clusters was based on the grazing type of animals. Following the collection of samples, they were immediately transferred to the Parasitology Laboratory of Faculty of Medicine, Lorestan University of Medical Sciences, Khorramabad, Iran. A part of each sample was kept at -20°C for molecular investigations and the rest was stored in the refrigerator for parasitological investigations.

2.2. Direct Investigation of Stool

To examine the stool samples, 30 ml of normal saline was poured into the fecal sample and kept at room temperature for 30 min. To remove large stool particles from the sample, specimens were mixed with normal saline and passed through a sieve. Subsequently, the mixture was poured into an Eppendorf tube and centrifuged at $2000 \times g$ for 4 min. A drop of sediment was placed on the slide and examined directly at three different magnifications of $\times 4$, $\times 10$, and $\times 40$ under a microscope.

2.3. Clayton-Lane Faecal Egg Flotation

The Clayton-Lane technique was performed in the manner described by Castelino and Herbert (1972). Briefly, each sample was made by mixing 5 g of faecal samples with 40 ml of water in a blender for approximately 2 min until a slurry was achieved. The mixed material was passed through a 3-inch-diameter CO mesh sieve, and after stirring, the filter was dissolved as a 15 mL solution in Clayton-Lane centrifuge tubes. Each sample was centrifuged at $2,500 g$ for 2 min and the supernatant was discarded. Saturated magnesium sulfate solution was added to each tube and the precipitate was thoroughly mixed before the tube was further diluted with magnesium sulfate solution to produce a positive meniscus. After

centrifugation, the number of recovered eggs was recorded (Castelino and Herbert, 1972).

2.4. Formalin Ethyl Acetate Concentration

A few grams of the fecal sample precipitate were passed through the sieve in a test tube, mixed with approximately 9 ml of 10% formalin, and incubated at room temperature for 30 min. Afterward, 2-3 ml of ethyl acetate was added to each one of the tubes, their caps were snapped, and each tube was shaken for 30 sec. To remove the gas produced in the tubes, their caps were gently removed and the samples were centrifuged at $2500 \times g$ for 4 min. Each tube consists of four layers (from bottom to top: fecal sample sediment, formalin, debris, and ethyl acetate). The debris layers were removed by an applicator and the rest of the content of the tubes, except the sediment, were discarded. A drop of sediment was placed on the slide and examined directly by a microscope at three different magnifications of $\times 4$, $\times 10$, and $\times 40$.

2.5. DNA Extraction

Specific kits were purchased from MBST Co. for extracting DNA from stool samples according to the instructions of the kit manufacturer. To break the *Fasciola* egg layers and facilitate the DNA extraction, at first, the sediment of each stool sample was passed through the sieve and sonicated 5 times at 90 volts, each time for 5 sec. Afterward, 300 μl of the sample was poured into 1.5 ml microtubes and DNA extraction was carried out according to the instructions of the Kit.

The eggs isolated from the uterus of several *Fasciola* flukes, obtained from infected animals in a slaughterhouse, were used as a positive control for sonication and DNA extraction procedures. Following each step of the 5-sec sonication, a drop of the sample was placed on the slide to check the amount of destruction of the eggs. Following the DNA extraction of the control samples, the concentration of the extracted DNA was also measured using a Nanodrop spectrophotometer.

2.6. Polymerase Chain Reaction

The intergenic spacer gene of *F. hepatica* was selected as the target gene for PCR assay. Table 1 shows the nucleotide sequences of all primers used in the PCR assay (Ai et al., 2010). The PCR was carried out in a final volume of 25 μ l, including 12.5 μ l of master mix (Ampliqon, Denmark), 2 μ l of primers (F and R), 2 μ l of extracted DNA, and 8.5 μ l of distilled water.

The extracted DNA was amplified in a thermal cycler (Bio-Rad, USA) as follows: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 45 sec, and

extension at 72 °C for 1 min. Eventually, the final extension was performed at 72 °C for 5 min. Negative-control samples (sterile water) were included in all PCRs. The PCR products were electrophoresed in a 1% TBE (Tris base–boric acid–EDTA) agarose gel and stained with DNA safe stain solution (1 μ g/ml). The PCR amplification is expected to produce 220 bp amplicons in a positive reaction.

2.7. Statistical Analysis

Statistical analysis was carried out by the calculation of Chi-square in SPSS software (version 19).

Table 1. Nucleotide sequences of the used primers for conventional polymerase chain reaction assay

Target	Nucleotide sequence	Amplicon
	F3-5'-CATTACCGACTCAGCTTGCA-3'	
Intergenic (AF179871)	spacer B3-5'-ACCAAACGTTCCGGTTAGGT-3'	220 bp

3. Results

3.1. Demographic Characteristics of the Studied Animals

Table 2 summarizes the demographic characteristics of the studied animals. In this study, the highest numbers of samples were collected from Khorramabad (n=38, 19.5%) and Aligodarz (n=27, 13.8%), in that order. Moreover, the lowest number of

samples were collected from Roomeshkan (n=7, 3.6%). It should also be mentioned that the majority of the studied animals were female (n=122, 62.60%). In addition, regarding the age of the studied animals, most of them were 3-4 years old (n=77, 39.5%) while the rest were 5-6 years old (n=46, 23.6%).

Table 2. Demographic characteristics of the studied animals

		N	%
	Khorramabad	38	19.5
	Aligodarz	27	13.8
	Poledokhtar	22	11.3
	Delfan	22	11.3
	Kouhdasht	18	9.2
	Selseleh	15	7.7
	Azna	13	6.7
Area of sampling	Boroujerd	12	6.2
	Chegeni	12	6.2
	Doroud	9	4.6
	Roomeshkan	7	3.6
Livestock gender	Male	73	37.4
	Female	122	62.6
Livestock age	1-2	72	36.9
	3-4	77	39.5
	5-6	46	23.6

3.2. Stool Examination Using Conventional Diagnostic Assays

Based on the results of the stool examination using conventional diagnostic assays, 4 (2.1%) out of 195 stool samples were positive for *F. hepatica* eggs (Table 3).

3.3. Polymerase Chain Reaction Assay

According to the results of gel electrophoresis of PCR products on 1% agarose gel stained with DNA safe stain, 7 (3.6%) out of 195 DNA samples extracted from sheep stool specimens were positive for *F. hepatica* (Figure 1) (Table 3). Furthermore, there was a statistically significant difference between these two methods (P=0.0421).

Table 3. Comparison of the results of polymerase chain reaction (PCR) and conventional diagnostic methods in terms of the detection of *Fasciola hepatica* in the studied sheep

Method	Number	%	P-value
Conventional diagnostic methods	4	2.1	
PCR technique	7	3.6	0.0421

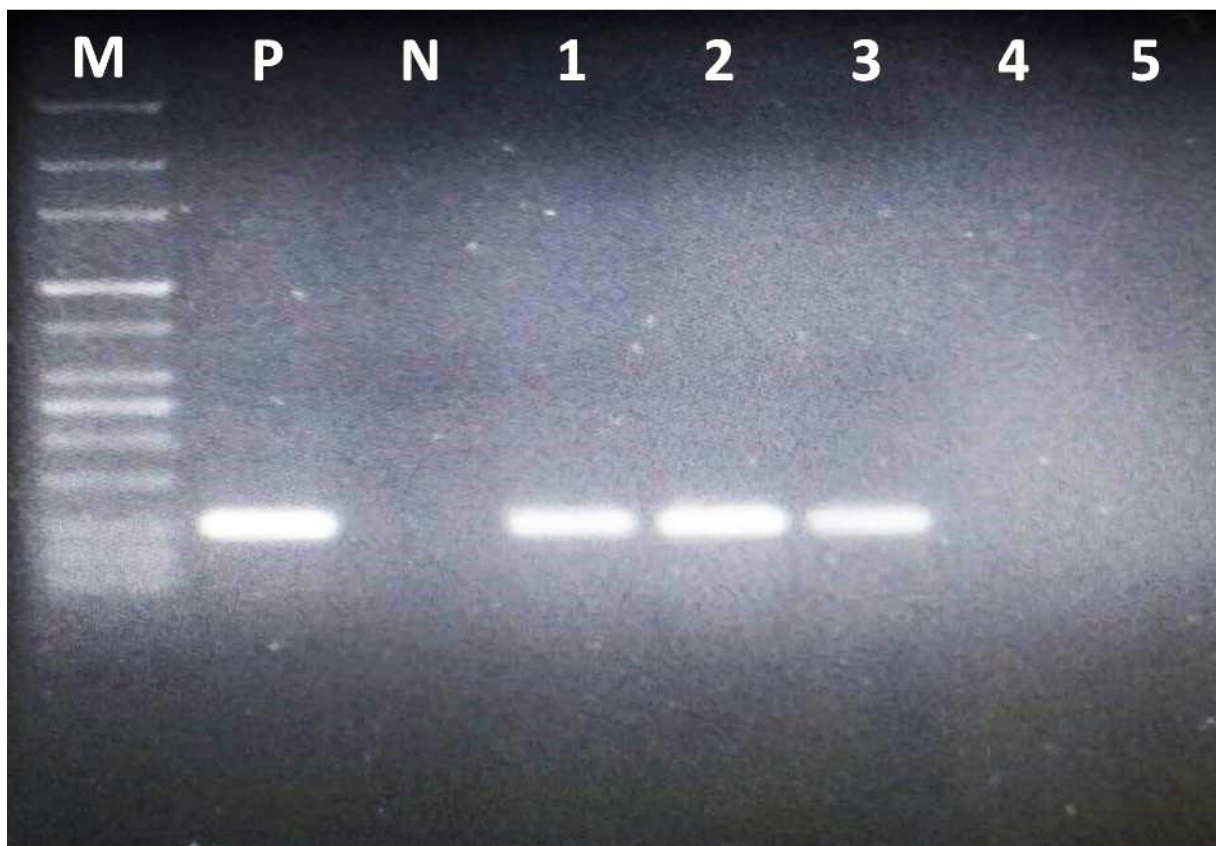


Figure 1. Polymerase chain reaction pattern of the gene pertaining to *Fasciola* spp. eggs from sheep for positive and negative samples.

M: 100bp DNA marker; P: positive control; N: negative control; 1-3: positive samples; 4 and 5: negative samples

4. Discussion

This study aimed to detect *F. hepatica* in Lori Sheep by PCR and routine parasitological methods. Fascioliasis is an important emerging food and water-borne disease in human communities and it is one of the most important health challenges in some countries, like Iran (Ghavami et al., 2009). Based on previous reports, two important human fascioliasis epidemics occurred in the 1990s in Guilan Province (Imani-Baran et al., 2012). The features of the disease had changed and its significance became evident (Asadian et al., 2013).

Different techniques have been used for the diagnosis of *F. hepatica*, and molecular techniques have been widely accepted recently. Based on the results of the present study, compared with other conventional techniques, the PCR can efficiently detect *F. hepatica* infection in Lori breed (seven positive samples by PCR vs. four positive samples by conventional assays).

Imani Baran et al. (2017) investigated the molecular diagnosis of *Fasciola* spp. isolated from fecal samples of domestic ruminants in the Northwest of Iran. They found that 72 (3.50 %) out of 2012 fecal samples were positive using microscopic examination of fecal samples of the cattle and sheep. They also found that PCR is an appropriate method for most epidemiological surveys.

Some studies have been performed to prove the presence of adult forms of *Fasciola* spp. in animals examined in slaughterhouses based on molecular diagnosis (Rokni et al., 2010; Shahbazi et al., 2011; Yakhchali et al., 2015) or determine the seroprevalence of fascioliasis in human communities (Rokni et al., 2010).

However, this study investigated eggs of *Fasciola* species of living ruminants in Iran. Khakpour and Garedaghi (2012) examined the molecular differentiation of sheep and cattle isolates of *F. hepatica* using RAPD-PCR and found that RAPD-PCR can efficiently detect *F. hepatica*, which is in line with the findings of the present study.

Ayaz et al. (2014) investigated *F. hepatica* in many buffaloes and cattle using PCR and microscopic examination and reported that the microscopic method could detect 3.58% of infected samples, which is similar to the results of this study. They also reported that PCR is a more sensitive diagnostic method, compared to microscopic examination. Similarly, in the present research, the diagnosis of *F. hepatica* was investigated in the West of Iran and it was found that the PCR technique was sensitive to the detection of *F. hepatica*.

5. Conclusion

In conclusion, this study was the first to detect *F. hepatica* in Lori sheep by PCR and routine diagnostic methods in Western Iran. The results indicated that PCR has more diagnostic sensitivity, compared to the concentration techniques and microscopic examination. Therefore, it can be advised to use PCR for the detection of *F. hepatica* in sheep.

Authors' Contribution

Study concept and design: B. Sh. and Sh. F.

Acquisition of data: S. A.

Analysis and interpretation of data: Sh. F.

Drafting of the manuscript: B. Sh. and Sh. F.

Critical revision of the manuscript for important intellectual content: S. Sh.

Statistical analysis: Sh. F.

Administrative, technical, and material support: S. A., B. Sh. and Sh. F.

Ethics

All used procedures were approved by the Ethical Committee of Islamic Azad University, Science and Research Branch, Tehran, Iran.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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