Detection of *Fasciola hepatica* in Lori Sheep using PCR and Conventional Diagnostic Methods in Western Iran

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ABSTRACT

Fascioliasis is an important emerging food and water-borne disease in human communities and it is one of the most important health challenges in countries like Iran. It causes weight loss, decrease in feed conversion ratio and milk and meat production as well as reduces fertility in animals. The prevalence of fasciolosis is increasing in some regions of the world due to many various factors. Different methods have been used for the detection of *Fasciola hepatica* (*F. hepatica*) in animals. This study was performed for the first time to detect *F. hepatica* in Lori sheep using polymerase chain reaction (PCR) and conventional diagnostic methods in western Iran. During 3 months, 195 fecal samples were collected from sheep in Lorestan province, West of Iran, by stratified random sampling method. The conventional diagnostic methods including wet mount microscopic examination
and concentration assays as well as the PCR technique targeting the IGS gene of *F. hepatica* were used for the detection of the parasite in sheep. Out of 195 examined stool samples, 4 (2.1%) samples were positive for *F. hepatica* by using 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). In sum, the results showed that PCR has more diagnostic sensitivity compared to conventional diagnostic methods including the concentration techniques and microscopic examination. It can be advised to use PCR for the detection of *F. hepatica* in sheep.

**Keywords:** Fascioliasis, Polymerase Chain Reaction, Conventional diagnostic methods

**INTRODUCTION**

*Fasciola hepatica*, *Fasciola gigantica*, and their hybrids cause fasciolosis that is an infectious disease and has huge global importance (Torgerson and Macpherson, 2011). It is estimated that fasciolosis causes US$2 billion loss annually (Calvani et al., 2018). It causes weight loss, decrease in feed conversion ratio and milk and meat production as well as 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 it is 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 many various factors including climate variations and environmental changes made by humans (Martínez-Valladares et al., 2013;
Rojo-Vázquez et al., 2012). Early and accurate detection of *Fasciola* species helps to control of fascioliasis effectively (Saki et al., 2011). Conventional methods are extensively used for the diagnosis of fascioliasis, but its diagnosis relies on the microscopic observation of *Fasciola* eggs in the fecal samples of infected animals (Shahzad et al., 2012). These methods are not only unable to diagnosis fascioliasis accurately and unable to identify intra-specific differences for *Fasciola* species, but also unable to identify eggs of different trematode (Robles-Péreza et al., 2013). Polymerase Chain Reaction (PCR) has recently become an interesting 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 from slaughtered animals in different regions of Iran, but their results have not been compared with the results of studies carried out using conventional methods (Rokni et al., 2010; Shahbazi et al., 2011; Yakhchali et al., 2015). A lot of people in Lorestan province work as stockbreeders, and they are mostly breeding sheep. It was necessary to carry out a study on the diagnosis of *F. hepatica* in Lori sheep by PCR. Thus, 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.

**MATERIAL AND METHODS**

**Samples collection**

A total of 195 fecal samples were collected from sheep in Lorestan province, West of Iran, by stratified random sampling procedure for 3 months. The samples were collected from 11 cities including Khorraramabad, Aligoudarz, Poledokhtar, Delfan, Kouhdasht, Selseleh, Azna, Boroujerd, Chegeni, Doroud, Roomeshkan, their districts, and their villages. Cluster sampling was used for sample collection in
different urban areas and villages. The distribution of samples/clusters was based on the grazing type of animals. Following the collection of samples, the samples 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.

**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 minutes. 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 × g for 4 min. A drop of sediment was placed on the slide and examined directly at three different magnifications of ×4, ×10 and ×40 under a microscope.

**Clayton-Lane Faecal egg flotation**

The Clayton-Lane technique was performed in the manner described by Castelino and Herbert. Briefly, each sample was made from 5 g by mixing with 40 ml of water in the blender for approximately 2 minutes in a slurry. 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 minutes and the supernatant was discarded. Saturated magnesium sulfate solution is added to each tube and the precipitate is thoroughly mixed before the tube is further diluted with magnesium sulfate.
solution to produce a positive meniscus. After centrifugation, the number of eggs recovered was recorded (Castelino and Herbert, 1972).

**Formalin ethyl acetate concentration**

A few grams of the fecal sample precipitate were passed through the sieve in a test tube and mixed with approximately 9 ml of 10% formalin and incubated at the room temperature for 30 minutes, and then 2-3 ml of ethyl acetate was added to each tube. The caps were snapped and each tube was shaken for 30 seconds. Then, to remove the gas produced in the tube, the tube cap removed gently and then the samples were centrifuged at 2500 × g for 4 minutes. Each tube consists of 4 layers (from bottom to top: fecal sample sediment, formalin, debris, and ethyl acetate). The debris layer was removed by an applicator and then all the contents of the tube 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 ×4, ×10 and ×40.

**DNA extraction**

Specific kits were purchased from MBST Co for extracting DNA from stool samples according to the instructions of the kit manufacturer. For breaking the *Fasciola* egg layers and facilitating the DNA extraction at first the sediment of each stool sample was passed through the sieve in the first step, was sonicated at 90 volts and 5 s for 5 times. Then, 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. 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 5 seconds sonication, a
drop of the sample was positioned 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.

**PCR**
The IGS gene of *F. hepatica* was selected as the target gene for PCR assay. The nucleotide sequences of all primers used in the PCR assay were shown in Table 1 (Ai et al., 2010). PCR was carried out in a final volume of 25 µl including 12.5 µl of master mix (Ampliqon, Denmark), 2µl of primers (F and R), 2 µl of extracted DNA and 8.5 µl 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 s, and extension at 72 °C for 1 min. 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 µg/ml). The PCR amplification is expected to produce 220 bp amplicons in a positive reaction.

**Statistical analysis**
Statistical analysis was carried out by the calculation of Chi-square in SPSS version 19.
RESULTS

Demographic characteristics of the studied animals
The demographic characteristics of the studied animals are shown in Table 2. In this study, the highest numbers of samples were collected from Khorramabad (n=38, 19.5%) and Aligodarz (n=27, 13.8%) respectively. Also, the lowest number of samples were collected from Roomeshkan (n=7, 3.6%). The majority of studied animals were female (n=122, 62.60%). Based on the age of studied animals, the highest number of animals were among 3-4 years old animals (n=77, 39.5%) and the lowest number of animals were among 5-6 years old animals (n=46, 23.6%).

Stool examination using conventional diagnostic assays
The results of stool examination using conventional diagnostic assays showed that out of 195 stool samples, 4 samples (2.1%) were positive for F. hepatica eggs (Table 3).

PCR assay
Gel electrophoresis of PCR products on 1% agarose gel, stained with DNA safe stain showed that out of 195 DNA samples extracted from sheep stool specimens, 7 samples (3.6%) were positive for F. hepatica (Fig. 1) (Table 3). And there was a statistically significant difference between these two methods (P=0.0421).

DISCUSSION
This study was carried out 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 epidemic occurred in the 1990s in Guilan Province (Imani-Baran et al., 2012). The feature of the disease had changed and its significance became evident (Asadian et al., 2013). Different techniques have been used for diagnosis of *F. hepatica*, but molecular techniques have been widely accepted recently. The results showed that compared with other conventional techniques, the PCR can efficiently detect *F. hepatica* infection in Lori breed (7 positive samples by PCR vs 4 positive samples by conventional assays). Imani-Baran et al. (2016) investigated the molecular diagnosis of *Fasciola* spp. isolates from domestic ruminant’s fecal samples in the Northwest of Iran and showed that out of 2012 fecal samples, 72 samples (3.50 %) were positive by microscopic examination of fecal samples of cattle and sheep. They also showed that PCR is an appropriate method for most epidemiological surveys. Some studies have been done to prove the presence of adult forms of *Fasciola* species 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; Saberinasab et al., 2014; Sarkari et al., 2012). However, this study investigated eggs of *Fasciola* species of living ruminants in Iran. Parallel to our results, Garedaghi and Khapkour (2012) investigated the molecular differentiation of sheep and cattle isolates of *F. hepatica* using RAPD-PCR and showed that RAPD-PCR can efficiently detect *F. hepatica*. Ayaz et al., (2014) investigated *F. hepatica* in many buffaloes and cattle by PCR and microscopic examination and reported that the microscopic method could detect 3.58% of infected samples, which is similar to our findings. They also reported that PCR is a more sensitive diagnostic method compared to microscopic examination. This study investigated the diagnosis of *F. hepatica* in the West of
Iran and showed that the PCR technique is sensitive to the detection of *F. hepatica*, as well.

**Conclusion**

In sum, this study was conducted for the first time to detect *F. hepatica* in Lori sheep by PCR and routine diagnostic methods in western Iran. The results showed that PCR has more diagnostic sensitivity compared to the concentration techniques and microscopic examination. It can be advised to use PCR for the detection of *F. hepatica* in sheep.

**Acknowledgments**

The authors would like to thank everyone who helped us with this study.

**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.

**References**


PCR- RFLP method based on internal transcribed spacer (ITS1, 5.8S rDNA, ITS2). Iranian J Parasitol 6, 35-42.


**Figure legends:**
Figure 1 PCR pattern of 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.

<table>
<thead>
<tr>
<th>Target</th>
<th>Nucleotide sequence</th>
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<td>F3-5’-CATTACCGACTCAGCTTGCA -3’</td>
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Table 2 Demographic characteristics of the studied animals

<table>
<thead>
<tr>
<th>Area of sampling</th>
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<td>13.8</td>
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<td>Poledokhtar</td>
<td>22</td>
<td>11.3</td>
</tr>
<tr>
<td>Delfan</td>
<td>22</td>
<td>11.3</td>
</tr>
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<td>Kouhdasht</td>
<td>18</td>
<td>9.2</td>
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<td>Selseleh</td>
<td>15</td>
<td>7.7</td>
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<td>6.7</td>
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<tr>
<td>Boroujerd</td>
<td>12</td>
<td>6.2</td>
</tr>
<tr>
<td>Chegeni</td>
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<td>6.2</td>
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<td>Doroud</td>
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<td>Roomeshkan</td>
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<td>Male</td>
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<td>37.4</td>
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<tr>
<th>Livestock gender</th>
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<td>62.6</td>
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<tr>
<td>Male</td>
<td>73</td>
<td>37.4</td>
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<th>Livestock age</th>
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<td>3-4</td>
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<td>5-6</td>
<td>46</td>
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</table>

Table 3 Comparison of the results of PCR and conventional diagnostic methods in detection of *Fasciola hepatica* in studied sheep

<table>
<thead>
<tr>
<th>Method</th>
<th>Number</th>
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<th>P-value</th>
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<tbody>
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<td>3.6</td>
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