DICROCOELIUM DENDRITICUM INFECTION AMONG LIVESTOCK IN WESTERN IRAN

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Abstract. Dicrocoelium, also known as liver fluke, is responsible for dicrocoeliasis in humans and many herbivores. Given the importance of medical, veterinary, health, and economics of dicrocoeliasis, the current study sought to identify D. dendriticum by PCR amplification of NADH dehydrogenase subunit gene (nad1) (900 bp) followed by Rsa-1digestion (206 and 694 bp) from liver of sheep, cows and goats (15 per group) in Ilam, Lorestan and Kermanshah Provinces, Iran. Of 45 D. dendriticum isolates identified (one per animal), 7 amplicons (900 bp) were verified by direct DNA sequencing.

Keywords: Dicrocoelium dendriticum; liver fluke, RFLP-PCR, Iran

INTRODUCTION

Dicrocoelium sp (Lancet liver fluke) is 6-10 mm in length and 0.2-0.4 mm in width and its size is directly proportional to the size of the final host (Beck et al., 2014). There are five species of parasites, namely, D. chinensis, D. dendriticum, D. hospes, D. orientalis, and D. supplereri, with D. dendriticum the most prevalent (Beck et al., 2015). There are two intermediate hosts and a final host in the life cycle of the parasite. The first intermediate host is terrestrial snail of the genus Helicella and Zebrina and the second intermediate host Formica ant, with the final host being mammals including herbivores (cattle, buffalo, sheep, goat, horse, deer, camel, rabbit, and wild ruminants), pig, rodents, polar bear, primates, and humans (Cabeza-Barrera et al., 2011). In the final animal host the flukes are located in the bile ducts, gallbladder and liver causing weight loss, slow growth, edema, anemia, and decreased milk production. The parasite causes fibrosis around the bile ducts and liver cirrhosis, which can lead to death (Otranto and Traversa, 2003; Abdi et al., 2013). Infection in humans is accidental and its symptoms are chronic diarrhea, constipation, abdominal pain, cramping, nausea, enlarged liver, pruritus, bile duct obstruction, and elevated eosinophils in peripheral blood (Stein et al., 2007).

Dicrocoeliasis is economically important because it results in a high cost of parasiticidal treatment and reduced livestock production, and in 2007 the World Health Organization classified dicrocoeliasis as a food-borne disease (Stein et al., 2007). Endemic dicrocoeliasis is distributed in 32 countries. D. dendriticum is the most prevalent species worldwide, being common in the Middle East, Europe, Asia, and North and South America (Beck et al., 2014; ibid, 2015). According to recent studies, dicrocoeliasis is
more common among livestock in north and northwest Iran (Manga-González and Gonzalez Lanza 2005; Daryani et al, 2006; Ghazani et al, 2008; Ahmadi et al, 2010). The prevalence of *D. dendriticum* varies from 0.026% in Fars Province to 36.21% in Guilan Province (Mohamadzadeh et al, 2016; Majidi-Rad et al, 2018).

However, there is no documentation to confirm the presence of dicrocoeliasis in western Iran. Because of the medical, veterinary and economic importance of dicrocoeliasis in Iran, particularly in the western region where livestock husbandry is highly concentrated, this study determined the presence of dicrocoeliasis and its causative agent among livestock in this region of Iran.

**MATERIALS AND METHODS**

**Geographical area**

The study was conducted in three provinces, Ilam, Kermanshah and Lorestan (Fig 1). Ilam Province is one of 31 provinces of western Iran bordering Iraq. It covers an area of 19,086 km² and includes the cities of Abdanan, Arkwaz, Darreh Shahr, Dehloran, Eyvan, Ilam, Mehran, and Sharable. Ilam Province shares its borders with three neighbor provinces, namely, Kermanshah in the north, Khuzestan in the south and Lorestan in the east, and Iraq in the west with 425 km of common border. In 2015, the population of Ilam Province is approximately 600,000, mainly nomads (Statistical Center of Iran, 2016). Kermanshah Province (34°18′N, 47°4′E), located in the center of the western region, consists of 14 areas: Dalaho, Gilan-e-gharb, Harsin, Islamabad-e-gharb, Javanrud, Kangavar, Kermanshah, Paveh, Qasr-e-Shirin, Ravansar, Sahneh, Sarpol-e-Zahab, Solas-e-Babajani, and Sonqor. The city of Kermanshah is built on the slopes of Mt Sefid Kooh and has extended towards the south during past two decades. The built-up areas run alongside Sarab River and Valley. The city’s elevation is about 1,350 m above sea level. It is the trade center of a rich agricultural region that produces grain, rice, vegetable, fruits, and oilseeds. There are many industrial centers, oil and
sugar refineries, and cement, textile, and flour factories. In 2015, the population of Kermanshah was 1,945,227 (Statistical Center of Iran, 2016). Lorestan Province with a population of 1,716,527 in 2006 is located in the Zagros Mountains. It covers an area of 28,392 km² consisting of 11 counties: Aligudarz, Azna, Borujerd, Delfan, Dorud, Doureh, Khorramabad, Kuhdasht, Poldokhtar, Rumeshkhan (as of 2013), and Selsele.

**Adult worm isolation**

After collecting infected livers from slaughtered animals, 45 putative adult *D. dendriticum* worms (one each from 15 cows, 15 sheep and 15 goats) were washed with normal saline solution, fixed in 70% ethanol and transported to the Laboratory of Parasitology, Ilam University of Medical Sciences.

**Restriction fragment length polymorphism (RFLP)-PCR analysis**

Worm samples were washed with phosphate-buffered saline (PBS) and DNA extracted using a commercial extraction kit (AccuPrep; Bioneer, Seoul, South Korea). PCR amplification was performed using primers Forward (5’-TTAAAA-CACACCCACCAGAA-3’) and Reverse (5’-GTGTTTTGGTTAATGTTTT-3’) targeting *D. dendriticum* NADH dehydrogenase subunit gene (*nad1*) (Gorjipoor et al, 2015) in a 2-µl reaction solution containing 5 µl of DNA, 1 µl of each primer (10 mM), 12 µl of Master Mix (Amplicon, Copenhagen, Denmark), and 6 µl of distilled water. Thermocycling was performed in a Bio-Rad T100™ thermocycler (Hercules, CA) as follows: 95°C for 5 minutes and 30 cycles of 95°C for 2 minutes, 58.4°C for 1 minute and 72°C for 2 minutes. Amplicons (900 bp) were separated by 1% agarose gel-electrophoresis and stained. Seven bands were randomly selected for direct DNA sequencing (Gen Fanavar, Tehran, Iran) and compared with GenBank *D. dendriticum* reference strain (accession no. NC_025280) using Chroma 4.2 software (Traverse City, MI). Then, 10 µl aliquot of PCR solution was added to a solution composed of 2 µl of 10X Fast Digest buffer (Fermentas, Vilnius, Lithuania), 1 µl of Fast Digest *Rsa*-1 and 18 µl of distilled water and incubated at 37°C for 40 minutes. Reaction products (206 and 694 bp) were analyzed by 1% agarose gel-electrophoresis and staining with DNA Safedye.

**RESULTS**

Forty-five putative *D. dendriticum*-infected liver samples based on macroscopic examination were collected from livestock (cows, sheep and goats) in different provinces of western Iran. PCR-RFLP then was carried out by amplifying *D. dendriticum*-specific *nad1* fragment (900 bp) followed by *Rsa*-1 digestion to yield 206 and 694 bp fragments. All samples were confirmed as *D. dendriticum* (Fig 2). In addition, DNA sequences of seven randomly chosen 900 bp *nad1* amplicons (LC218135.1) revealed 99-100% similarity to that of a reference *D. dendriticum* strain (GenBank accession no. NC_025280).

**DISCUSSION**

Although livestock husbandry is important in the western region of Iran no study hitherto has been carried out to identify *D. dendriticum* infection in this area. To the best of our knowledge, this is the first such study in livestock from the western provinces of Kermanshah, Ilam, and Lorestan. As only a limited number of samples were analyzed, it is possible that other *Dicrocoelium* spp might be detected in a larger survey. Maurelli et al (2007) employed 28S rDNA internal transcribed
spacer 2 (ITS-2) sequence to distinguish between *D. dendriticum* and *D. hospes*. Otranto *et al* (2007) revealed differences between *D. chinensis* and *D. dendriticum* specimens collected from different hosts and geographical localities though characterizing the sequences of a partial 18S rDNA fragment and ITS-2 (including 5.8S and 28S flanking regions). More recently, Arbabi *et al* (2012) employed RFLP-PCR of 28S rDNA to identify *D. dendriticum* in sheep and cattle.

Analysis of mitochondrial (mt)DNA has proven to be extremely useful in distinguishing within and among species of parasites because the mt genome is highly conserved but at the same time contains highly variable regions (Galtier *et al*, 2009). In addition, the mtgenome lacks recombination and rapid evolution. MtNad1 shows different sequence changes allowing a number of molecular techniques to be applied in genetic studies (Sabat *et al*, 2013). Several studies have been carried out on the genome of *Dicrocoelium* spp (Sandoval *et al*, 1999; Otranto and Traversa, 2002; Maurelli *et al*, 2007; Liu *et al*, 2014; Chang *et al*, 2016). In Iran, only one such study has been conducted by Gorjipoor *et al* (2015) in the province of Shiraz where they showed the presence of at least 10 and 2 distinct haplotypes in *D. dendriticum* nad1 and 28S rDNA ITS-2, respectively.

Sandoval *et al* (1999) employing random amplified polymorphic DNA (RAPD) profiling were able to demonstrate genetic variability among a population of *D. dendritic* infecting a single sheep, a variability which is similar among populations of worms from different sheep. On the other hand, the current study failed to note any genetic differences among single worms from the same and different mammalian hosts. Beck *et al* (2015) comparing experimental exposures in cattle and sheep with natural infection to determine recruitment, morphology and reproduction of adult *D. dendriticum* reported no

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**Fig 2- Electrophoregram of amplicons of Dicrocoelium dendriticum nad-1 (A) and Rsa-1 digested D. dendriticum nad-1 amplicon. A. Lane M, DNA size markers; lane 1, negative control; lanes 2-9, *D. dendriticum* DNA. B. Lane M, DNA size markers; lane 1, negative control; lanes 2-8, *D. dendriticum* nad-1 amplicon. C. Sizes of DNA markers.**
significant differences in recruitment of metacercariae and in pre-patency period of adult worms between the two groups of infected animals.

Awareness in Iran of the biological aspects of *D. dendriticum*, such as pathology, life cycle, morphology, control and treatment is poor. However, molecular methods, such as PCR, PCR-RFLP, RAPD-PCR, and sequencing, enable identification of *D. dendriticum* in the final or intermediate hosts. It is thus recommended that these molecular methods be more widely applied across the country.

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