DICROCOELIUM DENDRITICUM INFECTION AMONG LIVESTOCK IN WESTERN IRAN

Sahar Shokouhi[1,2], Asad Mirzaei[1], Razi Naserifar[1] and Jahangir Abdi[1]

[1]Department of Parasitology, Faculty of Medicine, [2]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran

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 0.2-0.4mm in width and its size is directly proportional to the size of the final host (Bec*k et al*, 2014). There are five species of parasites, namely, *D. chinensis, D. dendriticum, D. hospes, D. orientalis*, and *D. suppereri*, 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

Correspondence: Jahangir Abdi, Department of Parasitology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran.
Tel: +988432227134
E-mail: abdi-j@medilam.ac.ir

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 km^2 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 Lorestanin 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

Fig 1-Map of the three western provinces of interest in Iran.

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 km^2 constituting 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′-TTAAAACACACCCACCAGAA-3′) and Reverse (5′-GTGTTTTGGTTTAATGTTTT-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^{TM} thermocycler (Hercules, CA) as follows:95ºC for 5 minutes and 30 cycles of 95ºC for 2 minutes, 58.4ºC for 1 minute and 72ºC for 2 minutes. Amplicons (900 bp) were separated by1% agarose gel-electrophoresis and stained. Seven bands were randomly selected for direct DNA sequencing (Gen Fanavaran, 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 37ºC 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*-1digestion 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

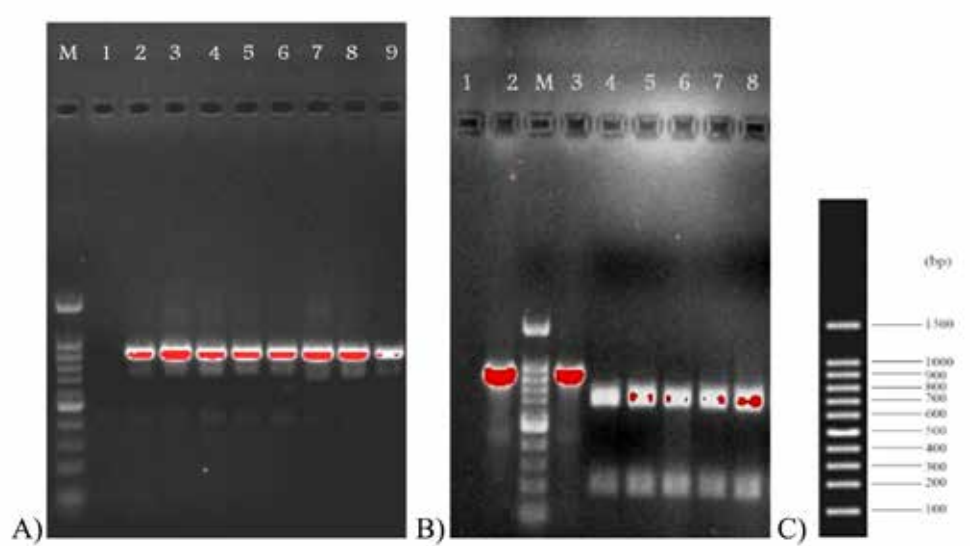

Fig 2- Electrophoregram of amplicons of *Dicrocoelium dendriticum nad-1* (A) and *Rsa-1* digested *D. dendriticum nad-1* amplicon. A. Lane M, DNA size markers; lane 1, negative control; lanes 2-9, *D. dendriticum* DNA. B. Lane M, DNA size markers; lane 1, negative control; lanes 2-8, *D. dendriticum nad-1* amplicon. C. Sizes of DNA markers.

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. Mt*Nad1*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

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.

ACKNOWLEDGEMENTS

The authors thank the Research Deputy of Ilam University of Medical Sciences for financial support.

REFERENCES

Abdi J, Karimi S, Naserifar R. Zoonotic parasitic diseases in Ilam Province, western Iran. *Afr J Microbiol Res* 2013; *7*: 2957-60.

Ahmadi R, Sikejor EM, Maleki M. Prevalence of *Dicrocoelium dendriticum* infection in cattle, sheep and goat in Gilan Province, northern Iran. *J Anim Vet Adv* 2010; *9*: 2723-4.

Arbabi M, Ghaffarifar F, Foorozandeh-Moghadam M. Morphological and molecular characterization of *Dicrocoelium* isolated from sheep in the north and center of Iran. *Feyz J Kashan Univ Med Sci* 2012; *16*:135-45.

Beck MA, Goater CP, Colwell DD. Comparative recruitment, morphology and reproduction of a generalist trematode, *Dicrocoelium dendriticum*, in three species of host. *Parasitology* 2015; 142: 1297-305.

Beck MA, Goater CP, Colwell DD, van Paridon, BJ. Fluke abundance versus host age for an invasive trematode (*Dicrocoelium dendriticum*) of sympatric elk and beef cattle in southeastern Alberta, Canada. *Int J Parasitol Parasites Wildl* 2014; *3*: 263-8.

Cabeza-Barrera I, Cabezas-Fernández T, Selas Coronas J, Vázquez Villegas J, Cobo F. *Dicrocoelium dendriticum*: an emerging spurious infection in a geographic area with a high level of immigration. *Ann Trop Med Parasitol* 2011; 105: 403-6.

Chang Q-C, Liu G-H, Gao J-F, *et al*. Sequencing and characterization of the complete mitochondrial genome from the pancreatic fluke *Eurytrema pancreaticum* (Trematoda: Dicrocoeliidae). *Gene* 2016; 576: 160-5.

Daryani A, Alaei R, Arab R, Sharif M, Dehghan M, Ziaei H. Prevalence of liver fluke infections in slaughtered animals in Ardabil province, northwestern Iran. *J Anim Vet Adv* 2006; 5: 408-11.

Galtier N, Nabholz B, Gkénub S, Hurst GD. Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Mol Ecol* 2009; 18: 4541-50.

Ghazani MHM, Valilou MR, Ahmadzadeh AR, Karami AR, Zirak K. The prevalence of sheep liver trematodes in the northwest region of Iran. *Turkish J Vet Anim Sci* 2008; *32*: 305-7.

Gorjipoor S, Moazeni M, Sharifiyazdi H. Characterization of *Dicrocoelium dendriticum* haplotypes from sheep and cattle in Iran based on the internal transcribed spacer 2 (ITS-2) and NADH dehydrogenase gene (*nad1*). *J Helminthol* 2015; *89*: 158-64.

Liu G-H, Yan H-B, Otranto D, *et al*. *Dicrocoelium chinensis* and *Dicrocoelium dendriticum* (Trematoda: Digenea) are distinct lancet fluke species based on mitochondrial and nuclear ribosomal DNA sequences. *Mol Phylogenet Evol* 2014; *79*: 325-31.

Majidi-Rad M, Meshgi B, Bokaie S. The prevalence and intensity rate of *Dicrocoelium dendriticum* infection in ruminants of 3 provinces in coastal regions of the Caspian Sea. *Iran J Vet Med* 2018; *12*: 27-33.

Manga-Gonzalez MY, Gonzalez-Lanza C. Field and experimental studies on *Dicrocoelium dendriticum* and dicrocoeliasis in northern Spain. *J Helminthol* 2005; *79*: 291-302.

Maurelli M, Rinaldi L, Capuano F, Perugini A,

Veneziano V, Cringoli G. Characterization of the 28S and the second internal transcribed spacer of ribosomal DNA of *Dicrocoelium dendriticum* and *Dicrocoelium hospes*. *Parasitol Res* 2007; 101: 1251-5.

Mohamadzadeh T, Shams S, Khanaliha K, Marhamatizadeh M, Vafa A. A study on prevalence of some helminthic infections of the liver and lungs among ruminants in abattoir of Fars province, Iran. *Arch Razi Inst* 2016; *71*: 245-51.

Otranto D, Rehbein S, Weigl S, *et al*. Morphological and molecular differentiation between *Dicrocoelium dendriticum* (Rudolphi, 1819) and *Dicrocoelium chinensis* (Sudarikov and Ryjikov, 1951) Tang and Tang, 1978 (Platyhelminthes: Digenea). *Acta Trop* 2007; 104: 91-8.

Otranto D, Traversa D. A review of dicrocoeliosis of ruminants including recent advances in the diagnosis and treatment. *Vet Parasitol* 2002; *107*: 317-35.

Otranto D, Traversa D. Dicrocoeliosis of ruminants: a little known fluke disease. *Trends Parasitol* 2003; *19*: 12-5.

Sabat A, Budimir A, Nashev D, *et al*. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveill* 2013; 18: 20380.

Sandoval H, Manga-Gonzalez Y, Campo R, Garcia P, Castro JM, de la Vega MP. Preliminary study on genetic variability of *Dicrocoelium dendriticum* determined by random amplified polymorphic DNA. *Parasitol Int* 1999; 48: 21-6.

Statistical Center of Iran. Population and Housing Censuss: Cencus 2016. Tehran: Statistical Center of Iran, 2016.

Stein C, Kuchenmuller T, Hendrickx S, *et al*. The global burden of disease assessments—WHO is responsible? *PLOS Negl Trop Dis* 2007; 1: e161.