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Molecular characterization and immunodiagnostics of *Dicrocoelium dendriticum* species isolated from sheep of north-west Himalayan region

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Research Paper

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Abstract

Despite its extensive presence among grazing ruminants, dicrocoeliosis, also known as 'small liver fluke' disease, is poorly known and often underestimated by researchers and practitioners in many countries. The accurate identification and prepatent diagnosis of *Dicrocoelium dendriticum* infection is an essential prerequisite for its prevention and control. In the present study, the morphologically identified specimens isolated from the bile ducts of sheep (*Ovis aries*) were validated through molecular data. The sequence analysis of the second internal transcribed spacer (ITS-2) of our isolates showed a high degree of similarity with *D. dendriticum* using the BLAST function of the National Center for Biotechnology Information (NCBI). The phylogenetic analysis of our isolates showed a close relationship with previously described *D. dendriticum* isolates from different countries. The antigenic profiles of somatic and excretory/secretory (E/S) antigens of *D. dendriticum* were revealed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting using sera from sheep naturally infected with *D. dendriticum*. By SDS–PAGE, 16 distinct bands were revealed from crude somatic fraction. Immunoblotting analysis of these proteins with positive sera exhibited six seroreactive bands ranging from 27 to 130 kDa. Among these, the 84 and 130 kDa bands were quite specific, with high diagnostic specificity and sensitivity. The E/S fraction comprised nine distinct bands, as revealed by SDS–PAGE analysis. Immunoblotting analysis of these proteins with positive sera exhibited five antigenic bands ranging from 27 to 130 kDa. Among these, the 130 kDa band was found to be quite specific, with high diagnostic specificity and sensitivity. The present study concludes that the protein bands of 84 and 130 kDa in somatic fraction and 130 kDa in E/S fraction can be used for the immunodiagnostic purpose for this economically important parasite, which may also encourage further studies regarding their vaccine potential.

Introduction

Dicrocoeliosis caused by *Dicrocoelium* spp. (small liver fluke) is a global parasitic disease of wide economic implications and public health concern (Gonzalez-Lanza *et al.*, 1993; Campo *et al.*, 2000). In India, limited information is available on the economic impact of this disease. The estimation becomes more complicated because dicrocoeliosis is often either not detected clinically or not diagnosed due to its subclinical nature (Ducommun & Pfister, 1991). The impact of parasitism on the livestock production also depends on the severity of infection, extent of the exposure, immunity of the host and the metabolic cost of a competent immune system. *Dicrocoelium dendriticum* shows cosmopolitan distribution throughout Asia, Europe, North Africa and North America (Soulsby, 1968). *Dicrocoelium dendriticum* parasitizes a number of hosts, particularly domestic and wild ruminants, but rabbits, pigs, dogs, horses and humans can also be infected occasionally (Otranto & Traversa, 2003). In addition to primary hosts, their distribution is contingent upon availability of intermediate hosts (snails and ants) and other environmental factors (temperature and humidity). The hepatic lesions caused by *D. dendriticum* often remain undetected as compared to lesions caused by other liver flukes. This is due to masking of its clinical signs by mixed infections of other more pathogenic helminths (Theodoridis *et al.*, 1991). In fact, it is difficult to investigate the pathogenesis of *D. dendriticum* under both natural and experimental conditions. The lack of evident lesions and clinical signs in the case of dicrocoeliosis may also be due to the behaviour of young flukes, which migrate directly up the biliary duct system of the liver without penetrating the gut wall or liver parenchyma (Theodoridis *et al.*, 1991). Studies related to pathogenesis under experimental conditions have shown a slight increase in bilirubin (7%) and albumin (3%), which is not, however, related to the worm burden (Otranto & Traversa, 2002; Manga-Gonzalez *et al.*, 2004). Under natural infections, the burden of up to 4000 adult flukes does not cause any significant loss in the blood and plasma proteins (Theodoridis *et al.*, 1991).

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However, cholangitis and cholangiectasia have been described in both natural and experimental infections (Ranucci *et al*., 1981; Wolff *et al*., 1984; Manga-Gonzalez *et al*., 2004). In the case of severe infections, necrotic and haemorrhagic tracts have been reported. Moreover, portal hepatitis and portal, septal and, on some occasions, perisinusoidal fibrosis have also been observed (Manga-Gonzalez *et al*., 2004). From the clinical aspect, the most significant sign of dicrocoeliosis is the reduced weight gain at the early stages of infection (Salimova, 1972; Manga-Gonzalez *et al*., 2004). However, the clinical signs are not pathognomonic, even in severe infections. The animals under severe conditions can develop anaemia, icterus, oedema and a slight reduction in production.

Because of its subclinical nature, dicrocoeliosis often remains clinically undiagnosed (Ducommun & Pfister, 1991). The most commonly used diagnostic technique includes the coprological examination for the detection of fluke eggs or recovering adults from the liver at necropsy (Otranto & Traversa, 2002). However, absence of eggs in the pre-patent phase (Nour Eldin *et al*., 2004) and intermittent release of eggs in the mature phase makes the results unreliable. The immunological methods for diagnosing dicrocoeliosis are based on the detection of antibodies in the serum against *D. dendriticum*. Various immunodiagnostic techniques like immunofluorescence (Calamel, 1977), passive haemagglutination test, complement fixation, enzyme-linked immunosorbent assay (ELISA) and Western blotting (Wedrychowicz *et al*., 1995, 1997; Gonzalez-Lanza *et al*., 2000) have been employed to detect anti-*Dicrocoelium* antibodies in naturally and/or experimentally infected animals.

Control of dicrocoeliosis is difficult and unsatisfactory because of the epidemiology and complex life cycle of its etiological agent. For the effective control of dicrocoeliosis, an accurate characterization of *D. dendriticum* at different taxonomic levels is essential. Although the morphological characteristics are used as the basis to identify and differentiate the species of *Dicrocoelium*, their accurate identification is difficult because of their close resemblance with closely related species. Thus, it becomes necessary to use alternative techniques, like the modern molecular tools and markers for their identification and differentiation (Gasser *et al*., 1999, 2008; Martinez-Ibeas *et al*., 2011; Huang *et al*., 2012; Wang *et al*., 2012). For this purpose, a number of genes have been used that include the sequences of the first internal transcribed spacer (ITS-1), the 5.8S and the second internal transcribed spacer (ITS-2) of the nuclear ribosomal DNA (rDNA), 28S ribosomal ribonucleic acid (rRNA) (Adlard *et al*., 1993; Itagaki & Tsutsumi, 1998; Marcilla *et al*., 2002; Itagaki *et al*., 2005a; Le *et al*., 2008; Ichikawa & Itagaki, 2010), 18S rRNA (Karimi, 2008), mitochondrial nicotinamide adenine dinucleotide (NAD) + hydrogen (H) dehydrogenase I (NDI) and cytochrome *c* oxidase I (COI) genes (Hashimoto *et al*., 1997; Itagaki *et al*., 2005b). The 18S and ITS-2 of rDNA have been employed for clear differentiation of *D. dendriticum* and *Dicrocoelium chinensis* (Otranto *et al*., 2007). Moreover, the 28S and ITS-2 rDNA have also been utilized to differentiate *D. dendriticum* and *Dicrocoelium hospes* (Maurelli *et al*., 2007). The larval stages of *D. dendriticum* in their intermediate hosts (snail and ant) were also detected based on their mitochondrial *cox* 1 and ITS-2 rDNA sequences (Martınez-Ibeas *et al*., 2011).

Keeping all this in view, the current study was designed to properly identify the species *D. dendriticum* and differentiate it from other species of *Dicrocoelium* using the morphological and molecular data. The study was also carried out to recognize the somatic and excretory/secretory (E/S) antigenic profile of *D. dendriticum* by immunoblotting technique using sera from sheep naturally infected with *D. dendriticum*.

Materials and methods

Collection and identification of parasites

Adult flukes of *D. dendriticum* were collected from the bile ducts of naturally infected Sheep (*Ovis aries*) slaughtered for consumption at the local slaughterhouses. Parasites were identified to species according to morphological features (Taira *et al*., 2006; Otranto *et al*., 2007).

Genomic DNA isolation, quality check and amplification

Genomic DNA was extracted using a NucleoSpin® Tissue Kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. The extracted DNA was treated with RNAase (100 mg/ml), split into aliquots and stored at −20°C until used. The quality of the isolated DNA was checked using agarose gel electrophoresis by using 0.8% of agarose gel in 0.5X Tris/Borate/EDTA (TBE) containing 0.5 µg/ml ethidium bromide.

Polymerase chain reaction (PCR) was used to amplify the nuclear ITS-2 of rDNA by using the primers CAS5p8sFt (forward: 5′-TGAACATCGACATTTYGAACGCATAT-3′) and CAS28sB1d (reverse: 5′-TTCTTTTCCTCCSCTTAYTTGATATG CTTAA-3′) (Ji *et al*., 2003). PCR amplification was carried out in a 20 µl reaction volume, which contained 1X Phire PCR buffer (contains 1.5 mM magnesium chloride), 0.2 mM each deoxyribonucleotide triphosphate (dNTP) {(deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP)}, 1 µl DNA, 0.2 µl PhireHotstart II DNA polymerase enzyme, 0.1 mg/ml Bovine Serum Albumin (BSA) and 3% Dimethyl sulfoxide (DMSO), 0.5 M betaine and 5 pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, California, U.S.A) with initiation at 95°C for 5 min, followed by 35 cycles including denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 50 s; a final extension step consisting of incubation at 72°C for 10 min was included. The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. The gels were visualized in an ultraviolet (UV) transilluminator (Genei, Banglore, India) and the image was captured under UV light using a gel documentation system (Bio-Rad, California, U.S.A).

Sequencing of the ITS-2 amplicons and phylogenetic analysis

After complete amplification of the DNA samples, the amplified products were sent to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala, for sequencing. BioEdit Sequence Alignment Editor (Hall, 1999) was used to trim and annotate the raw sequences. All the edited sequences were aligned in MEGA 7.0 (Kumar *et al*., 2016) using the clustalW algorithm (Thompson *et al*., 1994) in order to trace individual mutations. The sequences were identified using the BLAST function from the National Center for Biotechnology Information (NCBI), applying the default parameter. Additional sequences of *D. dendriticum* along with other species of *Dicrocoelium* from different geographical regions were retrieved from GenBank for sequence alignment and phylogenetic tree construction. Phylogenetic

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trees were constructed using the minimum-evolution method (Rzhetsky & Nei, 1992) in MEGA 7.0 (Kumar *et al.*, 2016) based on the Tamura and Nei model (1993).

Preparation of crude somatic and E/S fraction of proteins

Freshly collected samples were washed with ice-cold 0.01% phosphate-buffered saline PBS (pH 7.4) three times so as to reduce muscle constriction of the acetabulum and oral sucker, to avoid release of E/S products and enzymes of the parasites, as described by Anuracpreeda *et al.* (2009), with slight modifications. Protein extracts were obtained by homogenizing the flukes in ice-cold lysis buffer (0.01 M PBS, pH 7.2, containing 10 mM Tris hydrochloride, 150 mM sodium chloride, 0.5% Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF) and a 100 µl cocktail of protease inhibitors (Roche, Basel, Switzerland) using tissue homogenizer. Homogenization was carried out at 4°C for 10 min at 1300 rpm, taking pause of 2 min after every one minute of homogenization. After homogenization, the mixture was allowed to stand for half an hour on ice until the foam settled down. Thereafter, the suspension was sonicated for 10 min (pulse 10, lapse 30 and amplitude 70%) in an ice bath and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant protein fraction was concentrated using chilled acetone and aliquoted and stored at −20°C.

E/S proteins were prepared from living flukes according to Phiri *et al.* (2006), with slight modifications. The adult worms of *D. dendriticum* were collected from the bile ducts and washed three times in 0.01 M PBS, pH 7.4, at room temperature. The cleaned worms were then incubated (40 worms per 100 ml) in RPMI-1640 medium containing 2 mM PMSF, 100 IU of penicillin and 100 µg of streptomycin per millilitre of medium for about 3 h at 37°C. After incubation, the worms were removed from the medium and the suspension containing the E/S proteins of the worms was centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was collected, aliquoted and stored at −20°C. The protein concentrations of both fractions were measured according to Bradford (1976).

Collection of serum samples

Blood samples were collected from local abattoirs at the time of slaughtering of sheep with the monospecific infections of *D. dendriticum*. Blood samples were also obtained from uninfected sheep serving as the negative control. The infections were ascertained by post-mortem examinations. Sera were obtained from each clotted blood samples by centrifugation at 4000 rpm for 10 min and stored at −20°C.

Polypeptide analysis by *sodium dodecyl sulphate–polyacrylamide gel electrophoresis* (SDS–PAGE)

Somatic and E/S protein fractions of these liver flukes were separated by SDS–PAGE as described by Laemmli (1970). The protein samples were heated in a water bath at 100°C under denaturing and reducing conditions for 10 min. Samples were then loaded to each well of a 6% stacking gel and 12% separating gel and electrophoresis was carried out in Mini Protean II electrophoresis apparatus (Bio-Rad) at 90 V for about 4–5 h. Gels were stained with 0.05% Coomassie Brilliant Blue. The molecular weights of proteins were then determined by comparing their migration distance against that of a known molecular weight marker.

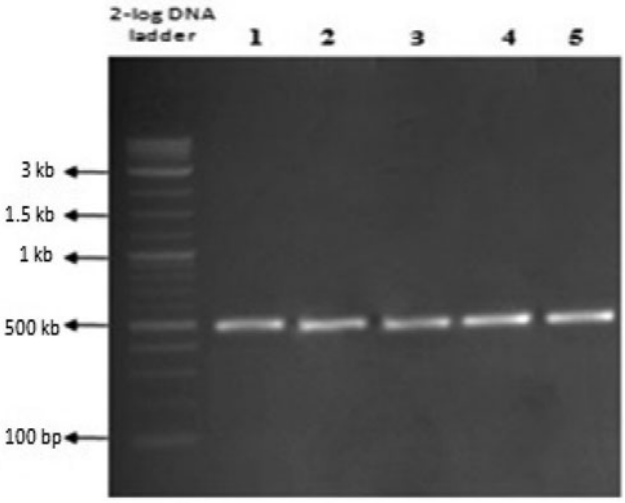

Fig. 1. Representative gel (1.2% agarose) image of amplified ITS-2 region of rDNA (L1–L5: *Dicrocoelium dendriticum*).

Antigenic analysis by Western blotting

Antigenically active components among the resolved bands in SDS–PAGE were detected by Western blotting. After SDS–PAGE, the unstained gels were transferred electrophoretically onto a nitrocellulose sheet using a transfer blot apparatus. Nitrocellulose sheets containing transferred sample strips were incubated in a blocking solution (1% skimmed milk and 0.1% Tween-20 in 100 mM PBS, pH 7.4) overnight at 4°C. The strips were then incubated with sera containing test antibodies. All sera were diluted 1:1000 in TBS and incubated at 4°C overnight, with gentle shaking. Following three PBS washes to remove unbound antibodies, the nitrocellulose sheets were then incubated for 1 h in horseradish peroxidase conjugate anti-Immunoglobulin-G antibodies. Unbound conjugate was removed by washing three times in PBS, before the addition of substrate solution containing 3,3′-diaminobenzidine. Gels were then visualized under the Bio-Rad gel documentation system.

Results

Molecular characterization

The amplified product of ITS-2 of *D. dendriticum* using the primers CAS5p8sFt (forward: 5′-TGAACATCGACATTTYGAAC GCATAT-3′) and CAS28sB1d (reverse: 5′-TTCTTTTCCTCCSC TTAYTTGATATGCTTAA-3′) yielded the fragment of 500 bp (fig. 1). The sequence analysis showed that ITS-2 rDNA had a length of 238 base pairs, and, along with ITS-2, were partial sequences of 5.8S and 28SrDNA sequences. The annotated sequence is shown in fig. 2, and the same sequences were submitted to NCBI via Banklt with accession numbers MH048705, MH753596, MH753597, MH753598 and MH753599.

The BLAST analysis of rDNA ITS-2 sequences JD1–JD5 isolates showed 98–100% similarity with *D. dendriticum*.

The phylogeny of *Dicrocoelium* species based on ITS-2 sequences was analysed using the minimum-evolution method. The present isolates formed a monophyletic clade with the already described species of *D. dendriticum* identified from different parts of the world. The bootstrap values are shown next to clades, and *D. hospes* (EF102026) was used as the outgroup taxa (fig. 3). The

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TTTTGGACATCGACATTTGAACGCATATTGCGGCCATGGGTTAGCCTGTGGCCACGC
CTGTCCGAGGGTCGGCTTACAAACTATCACGACGCCCAATAAGTCGTGGCTTGGATT
TTGCCAGCTGGCTTTACTCCCCAGTCGGAAACGTCAGGGGTGTCAGATCTATGGCGT
TATCCTAATGTATCCGGATACACACACCTAGTTATCAGACAGGTGGAGATGTGTCTA
CGGAGTCGTGGCTCAGTATTATTTATGCGCGCTCTGTAGAACATCTCGTGTGGTGAA
TTCCGAAATACGGCCTTCTAATCCTGACCTCGGATCAGACGTGATACCCGGCCGTC

Fig. 2. Annotated sequences of *Dicrocoelium dendriticum* isolate (5.8S rRNA gene, partial sequence (yellow); ITS-2, complete sequence (blue); and 28S rRNA gene, partial sequence (purple)).

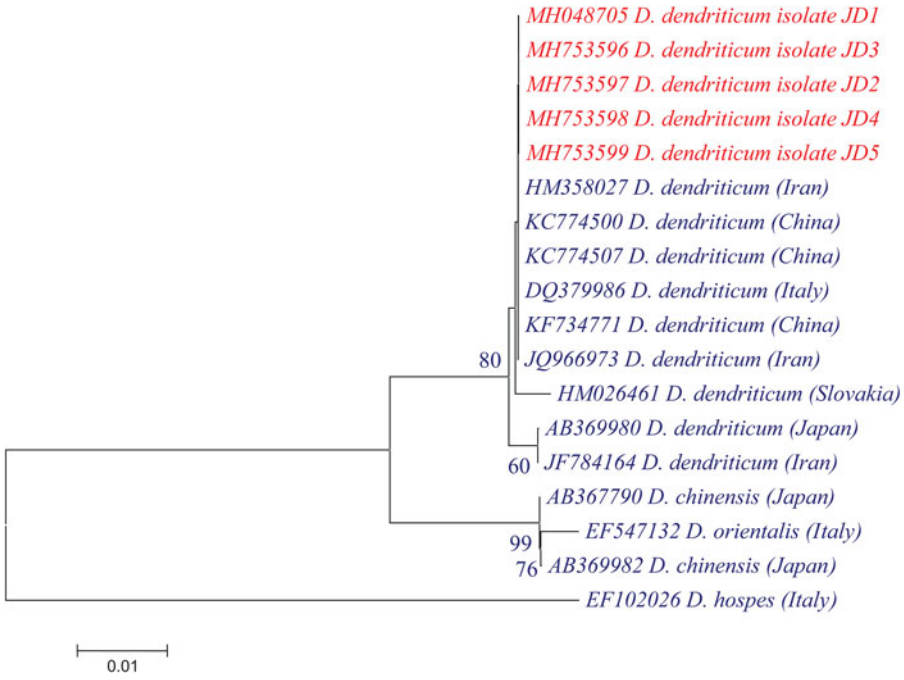

Fig. 3. Minimum-evolution phylogenetic tree, the bootstrap test (10,000 replicates), based on ITS-2 gene of *Dicrocoelium dendriticum* isolates, with *Dicrocoelium hospes* (EF102026) designated as outgroup. The analysis involved 18 nucleotide sequences. The evolutionary distances were computed using the Tamura–Nei method. All ambiguous positions were removed from each sequence pair. There were a total of 242 positions in the final dataset. Evolutionary analyses were conducted in MEGA 7.0. Scale bar shows the number of nucleotide substitutions per site.

Table 1. Comparison of sequence lengths and composition of ITS-2 between *Dicrocoelium* species from different parts of the world and present isolate JD1.

	Nucleotide composition										
Species	Accession number	ITS-2 (bp)	A + T (%)	G + C(%)	A	C	G	T	R	S	M
D. dendriticum JD1	MH048705	238	51.68	48.32	58	57	58	65			
D. dendriticum (Slovakia)	HM026461	241	51.45	48.55	57	59	58	67			
D. dendriticum (Iran)	HM358027	234	52.14	47.86	57	55	57	65			
D. dendriticum (Japan)	AB369980	239	51.88	47.7	58	57	57	66	1		
D. dendriticum (China)	KC774500	234	52.14	47.86	57	55	57	65			
D. dendriticum (Italy)	DQ379986	234	52.14	47.86	57	55	57	65			
D. orientalis (Italy)	EF547132	238	53.36	46.64	58	54	57	69			
D. hospes (Italy)	EF102026	233	53.65	46.35	63	55	53	62			
D. chinensis (Japan)	AB369982	238	52.94	46.22	58	54	56	68		1	1

sequence compositions of the ITS-2 regions of JD1–JD5 are 48.32% (GC content), 51.68% (AT content), A = 24.37% (58), C = 23.95% (57), G = 24.37% (58), T = 27.31% (65) (table 1). By comparing the ITS-2 sequences of our *D. dendriticum* isolates with the other species of *Dicrocoelium*, like *Dicrocoelium orientalis* and *D. chinensis*, the species were found to differentiate at 12 nucleotide sites, *viz.*: 1, 6, 57, 116, 131, 133, 135, 190, 201, 202, 209 and 232 (fig. 4).

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Fig. 4. Interspecies alignment of sequences of *Dicrocoelium dendriticum*, *Dicrocoelium orientalis* and *Dicrocoelium chinensis*, depicting variations at 12 positions in ITS-2 using the ClustalW alignment tool.

The distance matrix analysis carried out by *p*-distance and the maximum composite likelihood method of MEGA 7.0 showed 100% similarity and zero total character difference with sequences of already reported *D. dendriticum* isolates from various parts of the world. However, with other closely related species of the same genus, 7–26 bp differences were observed (table 2).

SDS–PAGE and immunoblotting

SDS–PAGE analysis of somatic protein extract of *D. dendriticum* revealed 16 protein bands between the molecular weights ranges of 15–175 kDa under reducing conditions (SDS–PAGE, 6% stacking and 12% resolving gel) (fig. 5). Western blotting analysis of somatic proteins with the sera of sheep naturally infected with *D. dendriticum* showed six bands with molecular weights of 27, 42, 66, 84, 97 and 130 kDa (fig. 6). Among these, the protein band of 130 kDa was most prevalent and was found to react with five out of six positive sera samples, while the 84 kDa band was found to react with four positive sera samples. No band was observed in the negative control sera.

The electrophoretic protein profile of E/S antigens of *D. dendriticum* under reducing conditions revealed nine protein bands with molecular weights ranging between 15 and 140 kDa (fig. 7). Western blotting analysis of E/S proteins with the sera of sheep naturally infected with *D. dendriticum* showed five bands with molecular weights of 27, 42, 50, 62 and 130 kDa (fig. 8). Among these, the protein band of 130 kDa was found to react with five out of six positive sera samples. No band was observed in the negative control sera.

Thus, protein bands with molecular weights of 130 and 84 kDa in somatic fraction and 130 kDa in E/S fraction of *D. dendriticum* were found to be highly specific.

Discussion

The accurate identification of *Dicrocoelium* species has an important implication for the epidemiology, control and management of dicrocoeliosis. The phylogenetic analysis of *Dicrocoelium* species based on their ITS-2 sequences using the minimum-evolution method showed that the isolates JD1–JD5 formed the clade with *D. dendriticum* isolates from different countries. ITS-2 sequences of *D. dendriticum* isolates in our study formed a single cluster with the *D. dendriticum* isolates from Slovakia, Italy, China and Iran, with significant bootstrap values. In the current study, ITS-2 was used as a genetic marker to identify the species of *Dicrocoelium* from the Kashmir Valley of India. ITS-2 sequences of *Dicrocoelium* isolates in our study were also compared with sequences of closely related species that exist in the GenBank database. According to the results obtained from the study, we concluded that *D. dendriticum* is the only species of *Dicrocoelium* that exists in the valley of Kashmir. No significant intraspecific variation was revealed among different isolates of *D. dendriticum*. However, considerable variation in the nucleotide sequence of ITS-2 was revealed when compared with the closely related species of *Dicrocoelium* from other parts of the world. For example, by comparing the ITS-2 sequences of our *D. dendriticum* isolates with the other species of *Dicrocoelium*, like *D. orientalis* and *D. chinensis*, the species were found to differ

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Table 2. Distance matrix analysis of the ITS-2 region between *Dicrocoelium* species.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	MH048705 *D. dendriticum* isolate JD1		**0**	**0**	**0**	**0**	**0**	**0**	**1**	**1**	**1**	**7**	**7**	**8**	**26**
2	HM358027 *D. dendriticum* (Iran)	**100**		0	0	0	0	0	1	1	1	7	7	8	25
3	KC774500 *D. dendriticum* (China)	**100**	100		0	0	0	0	1	1	1	7	7	8	25
4	KF734771 *D. dendriticum* (China)	**100**	100	100		0	0	0	1	1	1	7	7	8	25
5	KC774507 *D. dendriticum* (China)	**100**	100	100	100		0	0	1	1	1	7	7	8	25
6	DQ379986 *D. dendriticum* (Italy)	**100**	100	100	100	100		0	1	1	1	7	7	8	25
7	JQ966973 *D. dendriticum* (Iran)	**100**	100	100	100	100	100		1	1	1	7	7	8	26
8	HM026461 *D. dendriticum* (Slovakia)	**100**	100	100	100	100	100	100		2	2	8	8	9	26
9	AB369980 *D. dendriticum* (Japan)	**100**	100	100	100	100	100	100	99		0	8	8	9	25
10	JF784164 *D. dendriticum* (Iran)	**100**	100	100	100	100	100	100	99	100		8	8	9	25
11	AB367790 *D. chinensis* (Japan)	**97**	97	97	97	97	97	97	97	97	97		0	1	26
12	AB369982 *D. chinensis* (Japan)	**97**	97	97	97	97	97	97	97	97	97	100		1	26
13	EF547132 *D. orientalis* (Italy)	**97**	96	96	96	96	96	97	96	96	96	100	100		27
14	EF102026 *D. hospes* (Italy)	**88**	88	88	88	88	88	88	88	88	88	88	88	87	

at 12 nucleotide sites, *viz.*: 1, 6, 57, 116, 131, 133, 135, 190, 201, 202, 209 and 232.

Consistent with our studies, Arbabi *et al.* (2012) failed to demonstrate any significant intraspecific variation in the rDNA among different isolates of *D. dendriticum*. Similarly, Maurelli *et al.* (2007) reported the ITS-2 intraspecific variation of less than 1% in different isolates of *D. dendriticum* from different hosts, while the authors reported a higher interspecific variation of 8.2–8.5% between *D. dendriticum* and *D. hospes*. Along the similar lines, in the case of different isolates of *D. dendriticum*, Otranto *et al.* (2007) reported a low intraspecific variation of about 1.3% in the ITS-2 region of rDNA, irrespective of host species (sheep and cattle) or localities (Italy and Germany). Our results were also in accordance with the studies of Bian *et al.* (2015), who also used ITS-2 along with ITS-1 and 5.8S for the accurate identification and estimation of genetic variation in *Dicrocoelium* isolates. The authors reported the lengths of ITS-1, 5.8S and ITS-2 as 749, 161 and 234 bp, respectively, and the species was identified as *D. dendriticum* from NCBI-BLAST search, as well as from phylogenetic analysis. Whilst the intraspecific sequence variation among the *D. dendriticum* species was found to be 0–0.5% for ITS-1 and 0–1.3% for ITS-2 rDNA, the interspecific variations between different species in genus *Dicrocoelium* in ITS-2 rDNA were found to be 3.4–12.3%.

In addition to nuclear rDNA, mitochondrial genes like COI and NDI have also been used for the proper identification and characterization of *Dicrocoelium* species. For example, Liu *et al.* (2014) differentiated the two species of lancet liver flukes as *D. dendriticum* and *D. chinensis* based on mitochondrial and nuclear rDNA sequences. Results from the sequence comparison

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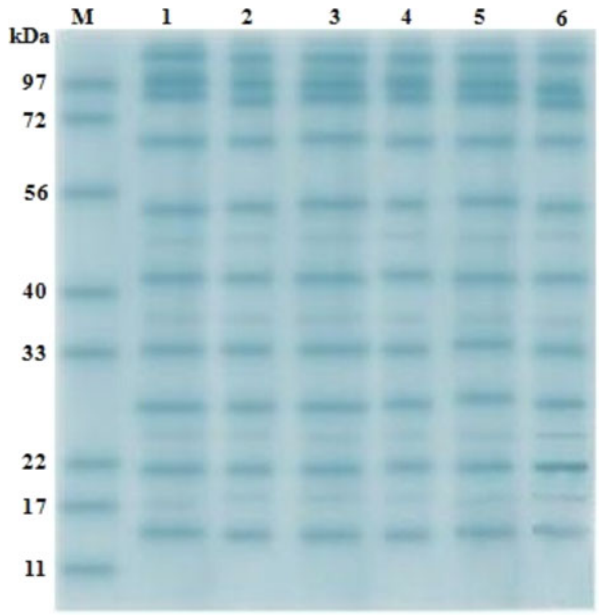

Fig. 5. Coomassie Brilliant Blue-stained protein profile of somatic antigens of *Dicrocoelium dendriticum* from different sheep livers (lanes L1–L6). M, molecular marker.

of a conserved mitochondrial gene and nuclear rDNA sequences among the number of lancet flukes revealed considerable nucleotide variation between the species but limited nucleotide variation within each of them.

The lower level of intraspecific nucleotide differences among the isolates of *D. dendriticum*, irrespective of host and locality, suggested a limited genetic affinity among the isolates of *D. dendriticum* and their host species. These findings accounted for a possible genetic stability within *D. dendriticum* population, as revealed for other generalist parasites, which infect multiple species of hosts all around the world (Humbert & Cabaret, 1995; Nolan & Cribb, 2005). However, it cannot be ruled out that a larger number of isolates sampled from different localities and host species may show a pattern of population subdivision not found in a more limited sample.

In our studies with *D. dendriticum,* SDS–PAGE analysis of somatic protein extract revealed 16 protein bands between the molecular weight range of 15–175 kDa under reducing conditions (SDS–PAGE, 10% stacking and 12% resolving gel), while the electrophoretic protein profile of E/S antigens of *D. dendriticum* under similar conditions revealed nine protein bands with molecular weights ranging between 15 and 140 kDa. Western blotting analysis of somatic proteins with the sera of sheep naturally infected with *D. dendriticum* showed six bands with molecular weights of 27, 42, 66, 84, 97 and 130 kDa. Among these, the protein bands of 84 and 130 kDa were found to react with four and five sera, respectively, out of six positive sera tested. No band was observed in the negative control sera. Western blotting analysis of E/S proteins with the sera of sheep naturally infected with *D. dendriticum* showed five bands with molecular weights of 27, 42, 50, 62 and 130 kDa. Among these, the protein bands of 130 kDa were found to react with five out of six positive sera samples tested.

Since there is limited information about the proteomic study of *D. dendriticum*, we have been able to make a lesser number of comparisons of our results with those of other researchers. By SDS–PAGE analysis, Revilla-Nuin *et al.* (2005) showed a greater number of polypeptides in both somatic (36) and E/S (18) antigenic fractions than our study in the case of *D. dendriticum*; however, most of their bands were similar to our study. Moreover, the authors also revealed eight immunoreactive antigenic polypeptides with molecular weights ranging from 24 to 205 kDa for somatic antigen and seven immunoreactive polypeptides between the molecular weight ranges of 26 and 205 kDa for E/S product of *D. dendriticum* using the immunoblotting technique. Our study supports and augments their study, as both the studies revealed the most specific immunoreactive band at the molecular weight of 130 kDa. This band also

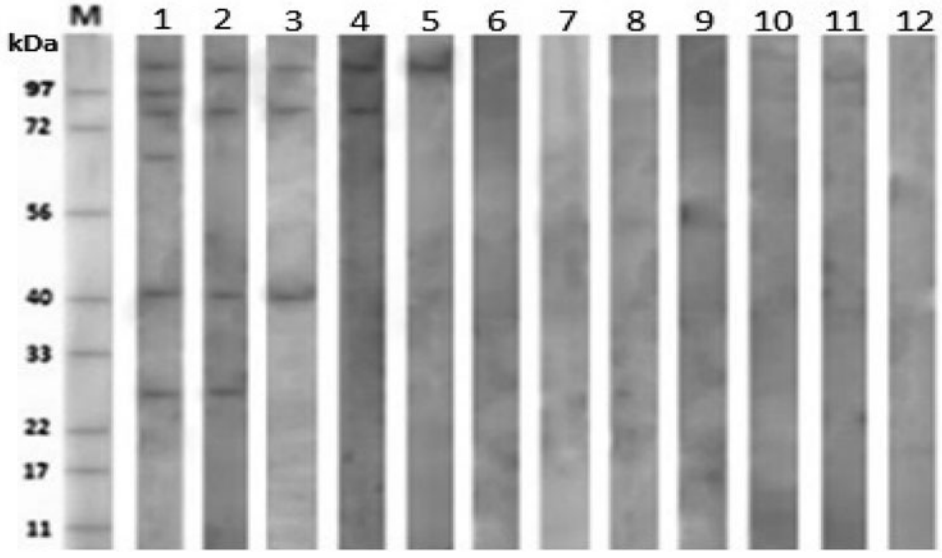

Fig. 6. Western blot analysis of somatic antigens of *Dicrocoelium dendriticum* against serum samples. M, molecular weight marker. 1–6 = positive sera of *D. dendriticum*; 7–12 = negative sera of *D. dendriticum*.

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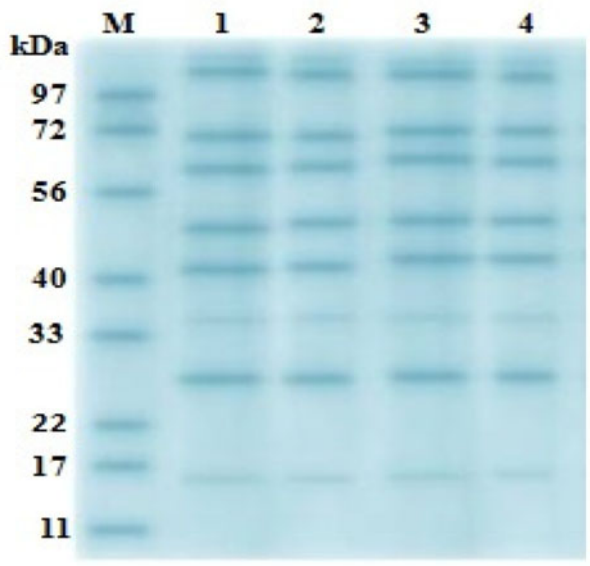

Fig. 7. Coomassie Brilliant Blue-stained protein profile of E/S antigens of *Dicrocoelium dendriticum* from different sheep livers (lanes L1–L4). M, molecular marker.

showed strong immunoreactivity by Western blot against ovine sera experimentally infected with *D. dendriticum* (Revilla-Nuin et al., 2005).

Revilla-Nuin et al. (2005) also carried out the partial sequencing of 130 kDa protein from both somatic and E/S products, revealing that both are identical at their amino-termini. In agreement with biochemical data, the comparison of this N-terminal sequence on database also revealed a perfect match (10/10 amino acids) with a peptide from *D. dendriticum* described as a globin (Kunz, 1975). This peptide, consisting of about 58 amino acids length, illustrates an enormous similarity with other peptides described as globins (Rashid *et al.*, 1997; Zhang *et al.*, 1997; Hu *et al.*, 2003; Sim *et al.*, 2003; unpublished results, see https://blast.ncbi.nlm.nih.gov/Blast.cgi, accession number AAX11352). Taking into consideration the aforementioned reports, Revilla-Nuin *et al.* (2005) postulated a possible relation of this 130 kDa protein with the family of globins. However, the protein band of 130 kDa from *D. dendriticum* is much larger than the described family of globins from other digenetic trematodes and also no homology was found for internal peptides. On the other hand, N-terminal sequences match properly with the amino-termini of the described Digenea globins. Thus, to summarize, the immunodiagnostic band of 130 kDa could be related to the family of globins, at least at its N-terminal end; however, more investigations are needed to establish its proper sequence and function.

Our studies were partially in accordance with Wedrychowicz *et al.* (1995) who, by using the SDS–PAGE technique, detected eight to nine polypeptides ranging from 29 to 205 kDa in the surface proteins and 17 bands in SDS soluble or somatic proteins of *D. dendriticum* extracted with TBS. Moreover, they detected 11–12 major polypeptides ranging between 15 and 205 kDa in culture fluids (E/S) collected before 18 h incubation, and only six polypeptides ranging from 15 to 78 kDa in those collected after 18–24 h. Our results regarding the immunodiagnostic bands of somatic and E/S antigens of *D. dendriticum* also partially coincide with those of Wedrychowicz *et al.* (1996), who revealed seven immunodiagnostic polypeptide bands between 28 and 124 kDa in the surface extracts and eight immunodiagnostic polypeptide bands between 21 and 158 kDa in the E/S product using Western blotting with rabbit antisera experimentally infected with *D. dendriticum*. Simsek *et al.* (2006) used SDS–PAGE analysis to reveal nine protein bands between the molecular weight ranges of 6 and 66 kDa with Coomassie stain and 14 bands with molecular weight ranges between 6 and 205 kDa with silver stain. Further, they revealed two immunodominant bands with molecular weights of 205 and 98 kDa in the E/S product of *D. dendriticum* using sera from positive animals, which was in disagreement with our results. Contrary to our studies, Meshgi & Khodaveisi (2014)

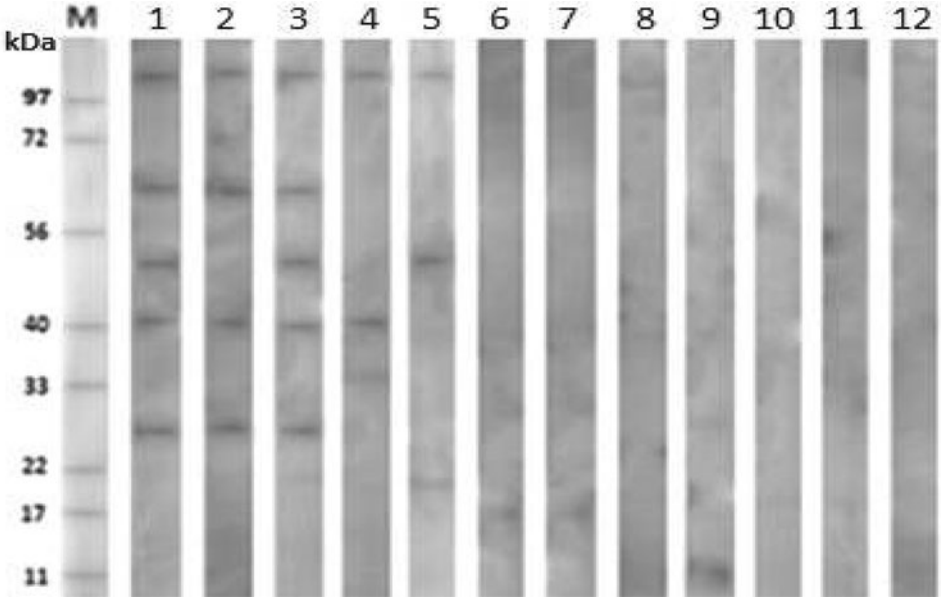

Fig. 8. Western blot analysis of E/S antigens of *Dicrocoelium dendriticum* against serum samples. M, molecular weight marker. 1–6 = positive sera of *D. dendriticum*; 7–12 = negative sera of *D. dendriticum*.

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showed a major immunoreactive band of 25–28 kDa in both somatic and E/S antigens of *D. dendriticum* using antisera from rabbits immunized with somatic and E/S antigens of *D. dendriticum*.

The present study concludes that the protein bands of 84 and 130 kDa in somatic fraction and 130 kDa in E/S fraction were found to give consistent reactions with the sera of infected sheep and can be used for the immunodiagnostic purpose for this economically important parasite. The present study also demonstrates the validity of the immunoblotting technique for the determination of *D. dendriticum* infections in sheep. However, the purification of these immunoreactive antigens may qualitatively improve the diagnostic value of such a test and may also encourage further studies regarding their vaccine potential.

E/S antigens have proved to be more useful for immunodiagnostic purpose as well as for protection against future infections than the somatic proteins (Parkhouse *et al*., 1987; Dalton & Heffernan, 1989). This is because E/S antigens more commonly get in touch with the host's immune system than somatic antigens. Most of the E/S antigens are the enzymatic proteins in the intestinal caeca of the parasite like cathepsins and others with cytolytic activities, and these enzymes are involved in degrading tissues and facilitating the invasion and migration of the parasite.

Different studies have shown a different number of protein bands using SDS–PAGE analysis, and a variable number and pattern of immunoreactive bands using Western blotting, which could be attributed to the existence of genetic variability in different isolates from different host species, geographical variations, the use of different extraction buffers in protein extraction processes and handling errors. Moreover, antigenic variations, as revealed by a number of helminths as a response to evade host immune response, may contribute to the variation in number and pattern of immunoreactive bands. The other reason could be the presence of both endogenous and host-derived proteins primarily during the isolation of flukes from thawed livers, as suggested by De Vera *et al*. (2009). Further, the differences can also be attributed to the existence of mixed trematode infections in the sheep, which may contribute to the observation of different immunogenic bands in different studies. Besides, the role of ecological and geographical factors cannot be neglected. Regardless of the difference existing in the number of protein bands or molecular weights of somatic and E/S polypeptides of *Dicrocoelium* species, the findings of various researchers suggest the existence of antigens with promising diagnostic and protective significance for humans and animals.

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Conflicts of interest. None.

Ethical standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

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