

Molecular characterization and Immunodiagnostic potential of various antigenic proteins of *Fasciola gigantica* species isolated from sheep of North West Himalayan Region

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Summary

The control of the digenetic trematode *Fasciola gigantica* has been the major challenge in both cattle and small ruminants as there is a paucity of an effective and commercial vaccine. Thus, the accurate identification and prepatent diagnosis of *F. gigantica* is an essential prerequisite for its successful prevention and control. In the present study, the morphologically identified specimens isolated from the liver and bile ducts of sheep (*Ovis aries*) were validated through molecular data. The sequence analysis of ITS-2 of our isolates showed high degree of similarity with *F. gigantica* and *F. hepatica* using BLAST function of NCBI. The phylogenetic analysis of our isolates showed a close relationship with previously described *F. gigantica* and *F. hepatica* isolates from different countries. The antigenic profile of somatic and E/S antigens of *F. gigantica* were revealed by SDS-PAGE and immunoblotting using sera from sheep naturally infected with *F. gigantica*. By SDS-PAGE, 20 distinct bands were revealed from crude somatic fraction. Immunoblotting analysis of these proteins with positive sera exhibited 8 sero-reactive bands ranging from 14 to 97 kDa. Among these 38 and 44 kDa bands were quite specific with high diagnostic specificity and sensitivity. The E/S fraction comprised 7 distinct bands, as revealed by SDS-PAGE analysis. Immunoblotting analysis of these proteins with positive sera exhibited 6 antigenic bands ranging from 23 – 54 kDa. Among these 27 and 33 kDa were found to be quite specific with high diagnostic specificity and sensitivity. The present study concludes that the protein bands of 38 and 44 kDa in somatic fraction and 27 and 33 kDa in E/S fraction can be used for the immunodiagnostic purpose for this economically important parasite, which may also entice further studies regarding their vaccine potential.

Keywords: *Fasciola gigantica*; ITS-2; BLAST; Excretory/Secretory antigen (E/S); Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE); Immunoblotting

Introduction

Livestock industry is faced with various types of bottlenecks that affect its production and parasitic infection is one of the important factors responsible for decline of livestock industry. A large groups of parasites like protozoans, trematodes, cestodes and nematodes are known to impinge on the livestock industry, but

trematodes have been found to be most disparaging, especially the gastrointestinal trematodes (Vercauteren & Claerebout, 2001; Roeber *et al.*, 2013). The members of the genus *Fasciola*, also known as liver flukes are responsible for causing a disease called ‘fasciolosis’ in livestock as well as humans. Because of their large size and cosmopolitan distribution, they have the global economic implications and are regarded as a concern for livestock and hu-

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man health. The economic loss incurred at the global level by fasciolosis infection is estimated to be about US\$ 3.2 billion annually which may be due to reduction of weight gain, draught tolerating capacity, fertility and lactation and costs associated for chemotherapy (Charlier *et al.*, 2008; Khan *et al.*, 2017). Equally important concern is the zoonotic transmission of these diseases to humans with 2.4 to 17 million people across world reported to be infected by fasciolosis and between 90 and 180 million known to be at risk of infection (Anonymous, 1995; Toledo *et al.*, 2011). Several studies have shown that *F. hepatica* and *F. gigantica* have unequal distribution. Whilst, *F. hepatica* is distributed throughout the world, *F. gigantica* has limited distribution and found only in areas of Asia and Africa. Further in the areas of geographical overlap, aspermic hybrid forms have developed making it difficult to differentiate between them. Such aspermic hybrids, which are thought to be the result of interspecific hybridization between the species of *F. hepatica* and *F. gigantica*, have been reported from Asia (Itagaki *et al.*, 2005a). Although the morphological characteristics are used as the basis to differentiate these two species of *Fasciola* (Ashrafi *et al.*, 2006), their accurate differentiation is difficult because at the extremes of their size range, individuals of one species may resemble with the other, with intermediate forms also occurring. Thus it becomes necessary to use alternative techniques like the modern molecular tools and markers for their identification and differentiation (Marcilla *et al.*, 2002; Periago *et al.*, 2004). For this purpose, a number of genes have been used which include the sequences of the first (ITS-1), the 5.8S and second (ITS-2) Internal Transcribed Spacers (ITS) of the nuclear ribosomal DNA (rDNA), 28S ribosomal ribonucleic acid (rRNA) (Adlard *et al.*, 1993; Itagaki and Tsutsumi, 1998; Marcilla *et al.*, 2002; Itagaki *et al.*, 2005a; Le *et al.*, 2008; Ichikawa and Itagaki, 2010), 18S rRNA (Karimi, 2008), mitochondrial NADH dehydrogenase I (NDI) and Cytochrome c Oxidase I (COI) genes (Hashimoto *et al.*, 1997; Itagaki *et al.*, 2005b). Further the use of novel single copy markers of nuclear genes like phosphoenolpyruvate carboxykinase (*pepck*) and DNA polymerase delta (*pold*) genes in the recent years have

been developed in this direction for the accurate discrimination of *F. hepatica*, *F. gigantica* and their hybrid forms (Shoriki *et al.*, 2015). Diagnosis of fasciolosis is an essential prerequisite for successful prevention and control of a parasitic disease. Efficient and state-of-the-art diagnostic tools and methods are important in the disease management program. Various tools ranging from traditional parasitological techniques to modern high-end molecular and immunological tools have been employed for the purpose. The most commonly used parasitological technique includes the fecal examination for the microscopic detection of liver fluke eggs by Kato-Katz test (Peters *et al.*, 1980). However, absence of eggs in the pre-patent phase and intermittent release of eggs in mature phase makes the results unreliable (Nour Eldin *et al.*, 2004). Moreover, in coproscopic detection of liver fluke infection faces limited accuracy and greater chances of false positive results (Dorchies, 2007). Therefore, early diagnosis is essential for effective treatment of the disease (Rokni *et al.*, 2004). In this direction various immunological detection techniques have been devised which are based on the presence of antibodies against the fluke antigens (Maleewong *et al.*, 1999). Such tests allow the pre-patent detection of the liver fluke disease before egg excretion. Other immunodiagnostic methods, involve the detection of fluke antigens inside the serum or other body fluids of infected host. Such tests are more accurate and allow early diagnosis than the antibody detection methods as the antigen is present in the host's serum or fluids before the presence of antibodies (Cornelissen *et al.*, 1999). However, these methods are also beset with many disadvantages, such as cross reactions with other trematode parasites, leading to false positive results (Hillyer *et al.*, 1985). The use of Western blotting/Immunoblotting techniques in recent years, have been able to overcome the chances of cross reactions to a greater extent. Of late, the emergence of proteomics has generated many biomarkers for efficient diagnosis of diseases. The identification and characterization of various proteins (mainly the candidates for immunodiagnosis or vaccination) over last two decades have been found to be of much immunological significance (Moxon *et al.*,

Table 1. Comparison of sequence lengths and composition of ITS2 rDNA of *Fasciola gigantica* from different parts of the world with our isolate JF1.

Species	Accession No.	ITS 2 (bp)	Nucleotide Composition					
			A+T (%)	G+C (%)	A	C	G	T
<i>F. gigantica</i> (JF1)	MH048702	362	51.93	48.07	71	80	94	117
<i>F. gigantica</i> (Iran)	JN828953	349	52.15	47.85	71	76	91	111
<i>F. gigantica</i> (Kenya)	KP760871	363	52.07	47.93	71	80	94	118
<i>F. gigantica</i> (India)	KX467878	365	51.51	48.49	71	82	95	117
<i>F. gigantica</i> (Australia)	MF678651	362	51.93	48.07	71	80	94	117
<i>F. gigantica</i> (Japan)	AB207152	363	51.79	48.21	71	81	94	117
<i>F. gigantica</i> (Indonesia)	AB010977	362	51.93	48.07	71	80	94	117
<i>F. gigantica</i> (Zambia)	AB010976	362	51.93	48.07	71	80	94	117
<i>F. gigantica</i> (Burkina Faso)	AJ853848	363	52.07	47.93	71	80	94	118

2010; Toledo *et al.*, 2011). Keeping all this in view, the current study was designed to properly identify the species of *F. gigantica* and differentiate it from *F. hepatica* by using the morphological and molecular data. The study was also carried out to recognize the somatic and excretory/secretory antigenic profile of *F. gigantica* by immunoblotting technique using sera from sheep naturally infected with *Fasciola gigantica*.

Materials and Methods

Collection of Parasites

Adult flukes of *F. gigantica* were collected from the liver and bile ducts of naturally infected Sheep (*Ovis aries*) slaughtered for consumption at the local slaughter houses. The samples for this study were collected during 2014 and 2015 from various slaughter houses of Kashmir valley. The collected flukes were washed 3 – 4 times with phosphate buffer saline (PBS) and used immediately for antigen preparation or stored in the laboratory at -20 °C until used.

Identification of the Parasite

The flukes were fixed in formalin, stained with hematoxylin and eosin, dehydrated in series of ascending grades of ethanol, cleared in xylene and mounted in DPX. The species were then identified morphologically according to Soulsby, 1982.

Genomic DNA Isolation, Quality check and Amplification

Genomic DNA was isolated from a piece of apical and lateral portions of adult *F. gigantica* using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. DNA was eluted in 50 µl of elution buffer (10 mM Tris, 1 mM EDTA) and kept at -20 °C until use. The quality of the DNA isolated was checked by Agarose gel electrophoresis using 0.8 % agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml ethidium bromide.

PCR was used to amplify the nuclear ITS-2 of ribosomal DNA by using the Primers BD1 (forward: 5'- GTCGTAACAAGGTTTC-CGTA -3') and BD2 (reverse: 5'- TATGCTTAAATTCAGCGGGT -3'). PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X Phire PCR buffer (contains 1.5 mM MgCl₂), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 1 µl DNA, 0.2 µl Phire Hotstart II DNA polymerase enzyme,

0.1 mg/ml BSA and 3 % DMSO, 0.5M Betaine, 5pM of forward and reverse primers. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with initiation at 94 °C for 5 min, followed by 35 cycles including denaturation at 94 °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 a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system.

Sequencing and Phylogenetic analysis

After amplification of the DNA samples, the amplified products were sent to Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, Kerala for sequencing. The sequences obtained from each primer were assembled and edited in Bioedit sequence alignment editor (Hall 1999). All the edited sequences were aligned in MEGA 7.0 [30], using the clustalW algorithm (Kumar *et al.*, 2016), in order to trace individual mutations. The sequences were identified using BLAST function from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Additional sequences of *F. hepatica* and *F. gigantica* from different geographical regions were retrieved from GenBank for sequence alignment and phylogenetic tree construction. Phylogenetic trees were constructed by Minimum Evolution method (Rzhetsky and Nei, 1992) in MEGA 7.0 (Kumar *et al.*, 2016) based on Tamura and Nei model.

Preparation of crude Somatic and excretory-secretory (E/S) proteins

Somatic proteins were extracted by the methodology described by Anuracpreeda *et al.*, (2009). Protein extracts were obtained by homogenization of 5 flukes in 5 ml of ice cold lysis buffer (0.01 M phosphate buffer saline (PBS), pH 7.2, containing 10 mM Tris-HCl, 150 mM NaCl, 0.5 % Triton X-100, 10 mM EDTA, 1 mM PMSF and 100 µl of cocktail of protease inhibitors) using tissue homogenizer. Homogenization was carried out for 10 minutes at 1300 rpm taking pause of 2 minutes after every one minute of homogenization. The supernatant protein fraction was concentrated using chilled acetone.

Table 2. Comparison of sequence lengths and composition of ITS2 rDNA of *Fasciola hepatica* from different parts of the world with our isolate JF4.

Species	Nucleotide Composition							
	Accession No.	ITS 2 (bp)	A+T (%)	G+C (%)	A	C	G	T
<i>F. hepatica</i> (JF4)	MH048706	363	51.79	48.21	70	80	95	118
<i>F. hepatica</i> (Australia)	MF678650	363	51.79	48.21	70	80	95	118
<i>F. hepatica</i> (Turkey)	JN585288	362	51.38	48.62	70	81	95	116
<i>F. hepatica</i> (Egypt)	AB553720	363	51.79	48.21	70	80	95	118
<i>F. hepatica</i> (Japan)	AB010978	362	51.66	48.34	70	80	95	117

Excretory-Secretory (E/S) proteins were prepared from living flukes according to Phiri *et al.*, (2006) with slight modifications. The adult worms of *F. gigantica* were collected from the liver and bile ducts and washed 3 times in 0.01 M phosphate buffered saline (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 phenylmethanesulfonyl fluoride (PMSF), 100 IU of penicillin and 100 µg of streptomycin per milliliter of medium for about 3 hours at 37 °C. After incubation, the worms were removed from the medium and the suspension containing the E/S proteins was centrifuged at 13000 rpm for 30 minutes 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 sera samples

Blood samples were collected from local abattoirs at the time of slaughtering of sheep with the mono-specific infections of *F. gigantica* and from uninfected negative control sheep. Blood sample was also collected from sheep infected with *Paramphistomum* spp. The infections were ascertained by Postmortem examinations and by coprological techniques (Thienpont *et al.*, 1979; Olaechea *et al.*, 1990). Sera were obtained from each clotted blood samples by centrifugation at 4000 rpm for 10 minutes, and stored at -20°C.

SDS-PAGE and Immunoblotting

Somatic and E/S proteins of *F. gigantica* were separated by SDS-PAGE as described by Laemmli (1970). The SDS-PAGE antigen separation was done under reducing conditions in gradient polyacrylamide gel cast with 12 % separating and 6 % stacking gel mixture. The electrophoresis was carried out in Mini Protean II electrophoresis apparatus (Bio-Rad, Hercules, CA) at 90 V for about 3 – 4 hours. 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 marker. After SDS-PAGE, the unstained gels were transferred electrophoretically onto a nitrocellulose sheet using a transfer blot apparatus. Nitrocellulose 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 3 PBS washes to remove unbound antibodies, the nitrocellulose sheets were then incubated for 1 hour in horseradish peroxidase conjugate anti-IgG antibodies. Unbound conjugate was removed by 3 PBS washes before the addition of substrate solution containing DAB (3,3'- Diaminobenzidine). Gels were then visualized under Bio Rad gel documentation system.

Evaluation of the diagnostic sensitivity of above antigens:

The diagnostic sensitivity, specificity, accuracy and predictive val-

ues of various antigens were tested with the sera from 12 sheep with natural infections of *Fasciola gigantica*, 6 uninfected hosts, together with 6 sera from sheep with *Paramphistomum* infection. Each of these parameters was calculated using following formulas:

$$\text{Sensitivity} = [A/(A+C)] \times 100$$

$$\text{Specificity} = [D/(B+D)] \times 100.$$

$$\text{Accuracy} = [(A+D)/(A+B+C+D)] \times 100$$

$$\text{Positive predictive value} = [A/(A+B)] \times 100.$$

$$\text{Negative predictive value} = [D/(C+D)] \times 100.$$

Where;

A= number of true positive,

B= number of false positive,

C= number of false negative,

D= number of true negative.

The primary data of immunoblotting are as follows:

True positive = number of proven infected samples that show positive result.

True negative = number of control samples (other parasitoses and healthy controls)

that show negative result.

False positive = number of control samples that show positive result.

False negative = number of proven infected samples that show negative result.

Ethical approval

The conducted research is not related to either human or animal use.

Results

Morphological identification of *F. gigantica* was carried out on the basis of size and shape of fluke, presence of shoulders and position of anterior and posterior sucker. Adult flukes of *F. gigantica* were found to be larger in size (up to 60 mm) than *F. hepatica*. The body shape was found to be dorso-ventrally flat or leaf

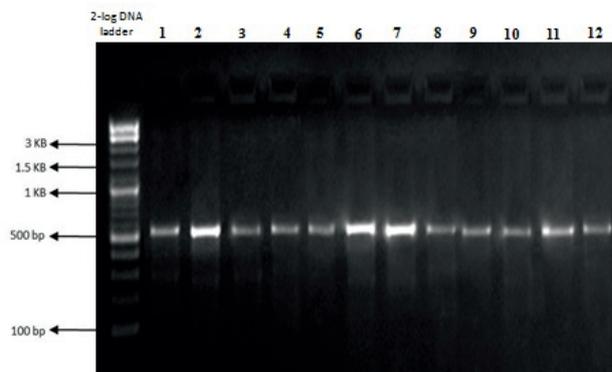


Fig. 1. Agarose gel electrophoresis of ITS-2 rDNA. Lanes 1 – 7 (*F. gigantica*) and lanes 8 – 12 (*F. hepatica*).

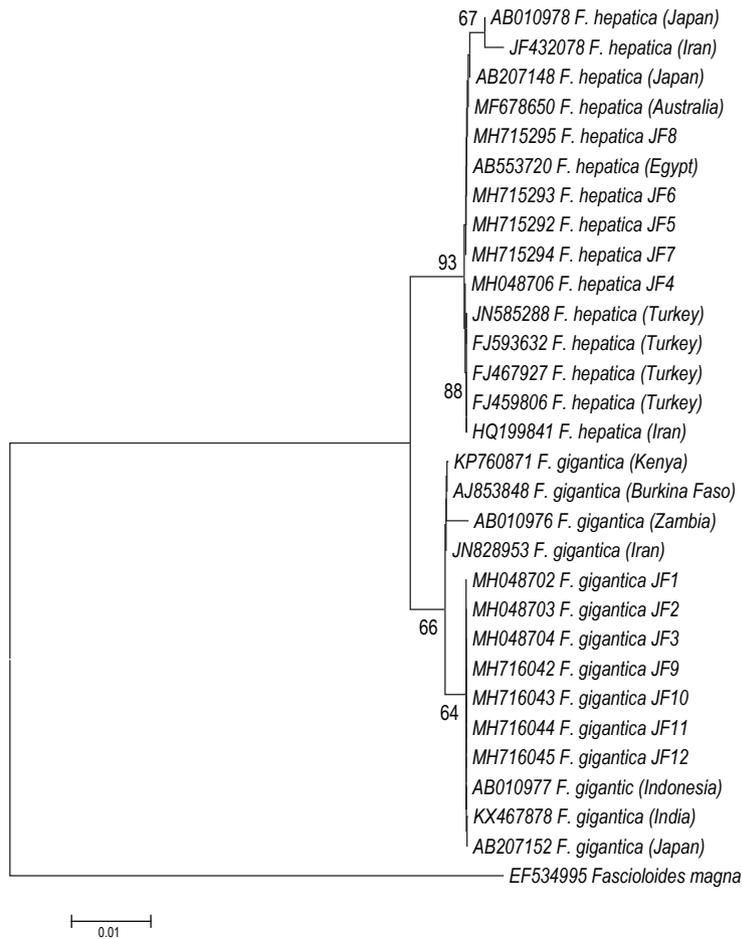


Fig. 2. Minimum Evolution phylogenetic tree, the bootstrap test (10000 replicates), based on ITS-2 gene of *F. gigantica* and *F. hepatica*, *Fascioloides magna* (EF534995) designated as outgroup. The analysis involved 30 nucleotide sequences. All ambiguous positions were removed from each sequence pair. There were a total of 367 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

like and possess conical anterior end followed by the shoulders. The body was found to be covered by the tegument armored with backwardly projecting spines. Ventral sucker also known as acetabulum was found to be larger than the oral sucker.

Molecular Characterization

During the present investigation, liver fluke species viz. *Fasciola gigantica* and *F. hepatica* were obtained and molecular data confirmed and validated the morpho-taxonomic characterization. Among these, seven isolates viz JF1-JF3, JF9-JF12 were found close to topotype population of *Fasciola gigantica* and five isolates (JF4-JF8) showed similarity with *F. hepatica*. The PCR amplification of ITS-2 using Primers BD1 (forward: 5'- GTCGTAACAAGGT-TTCCGTA -3') and BD2 (reverse: 5'- TATGCTTAAATTCAGCGG-GT -3') yielded fragments of 550 bp for both the species of *Fasciola* (Fig. 1). The sequence analysis showed that ITS2 rDNA had a length of 362 base pair in case of *F. hepatica* isolates and 363 base pair in *F. gigantica* isolates and, along with ITS2 were partial sequences of 5.8s and 28s rDNA sequences. The annotated

sequences of these isolates were submitted to NCBI via BankIt with accession numbers: MH048702, MH048703, MH048704, and MH716042-MH716045 for seven *F. gigantica* isolates, MH048706 and MH715292-MH715295 for five *F. hepatica* isolates. The BLAST analysis of rDNA ITS2 sequences of JF1-JF3, JF9-JF12 isolates showed 97 – 100 % similarity with *F. gigantica*, while the sequences of ITS2 rDNA of JF4-JF8 isolates showed 97 – 100 % similarity with *F. hepatica*.

Phylogenetic trees were constructed by comparing ITS2 sequences of this study with those of other liver fluke species deposited in the GenBank from across the world. Minimum evolution tree was used to construct phylogeny. *F. gigantica* and *F. hepatica* were separated in two clusters. Reliable grouping among ITS2 sequences of *F. gigantica* and *F. hepatica* from the current study and those from Australia, Indonesia, Japan, Burkina Faso, Kenya, Zambia, Iran, Egypt, Turkey and India are shown in Fig. 2.

The phylogenetic analysis of the *Fasciola* species based on ITS-2 regions by Minimum evolution tree showed a clear monophyly of the group formed by the present *F. gigantica* isolates and previ-

Table 3: Pairwise distances of the ITS-2 region of *Fasciola* Spp. from Kashmir India, compared with other sequences submitted to GenBank...

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1 MH048702 <i>F. gigantica</i> isolate JF1		0	0	0	0	1	1	1	2	5	5	5	5	5	5	5	5	5	6	39
2 KX467878 <i>F. gigantica</i> (India)	100		0	0	0	1	1	1	2	5	5	5	5	5	5	5	5	5	8	39
3 MF678651 <i>F. gigantica</i> (Australia)	100	100		0	0	1	1	1	2	5	5	5	5	5	5	5	5	5	6	39
4 AB207152 <i>F. gigantica</i> (Japan)	100	100	100		0	1	1	1	2	5	5	5	5	5	5	5	5	5	7	39
5 AB010977 <i>F. gigantica</i> (Indonesia)	100	100	100	100		1	1	1	2	5	5	5	5	5	5	5	5	5	6	39
6 KP760871 <i>F. gigantica</i> (Kenya)	100	100	100	100	100		0	0	1	4	4	4	4	4	4	4	4	4	8	39
7 JN828953 <i>F. gigantica</i> (Iran)	100	100	100	100	100	100		0	1	4	4	4	4	4	4	4	4	4	4	37
8 AJ853848 <i>F. gigantica</i> (Burkina Faso)	100	100	100	100	100	100	100		1	4	4	4	4	4	4	4	4	4	6	39
9 AB010976 <i>F. gigantica</i> (Zambia)	100	100	100	100	100	100	100	100		5	5	5	5	5	5	5	5	5	6	40
10 MH048706 <i>F. hepatica</i> isolate JF4	99	99	99	99	99	99	99	99	99		0	0	0	0	0	0	0	0	1	39
11 MF678650 <i>F. hepatica</i> (Australia)	99	99	99	99	99	99	99	99	99	100		0	0	0	0	0	0	0	1	39
12 JN585288 <i>F. hepatica</i> (Turkey)	99	99	99	99	99	99	99	99	99	100	100		0	0	0	0	0	0	3	39
13 FJ593632 <i>F. hepatica</i> (Turkey)	99	99	99	99	99	99	99	99	99	100	100	100		0	0	0	0	0	3	39
14 FJ467927 <i>F. hepatica</i> (Turkey)	99	99	99	99	99	99	99	99	99	100	100	100	100		0	0	0	0	3	39
15 FJ459806 <i>F. hepatica</i> (Turkey)	99	99	99	99	99	99	99	99	99	100	100	100	100	100		0	0	0	3	39
16 AB553720 <i>F. hepatica</i> (Egypt)	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100		0	0	1	39
17 AB010978 <i>F. hepatica</i> (Japan)	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100		0	0	39
18 HQ199841 <i>F. hepatica</i> (Iran)	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100		3	39
19 JF432078 <i>F. hepatica</i> (Iran)	99	99	99	99	99	99	99	99	99	100	100	100	100	100	100	100	100	100		41
20 EF534995 <i>Fascioloides magna</i>	93	93	93	93	93	93	93	93	93	93	93	93	93	93	93	93	93	93	93	100

Below diagonal: percentage similarity; above diagonal: total character differences.

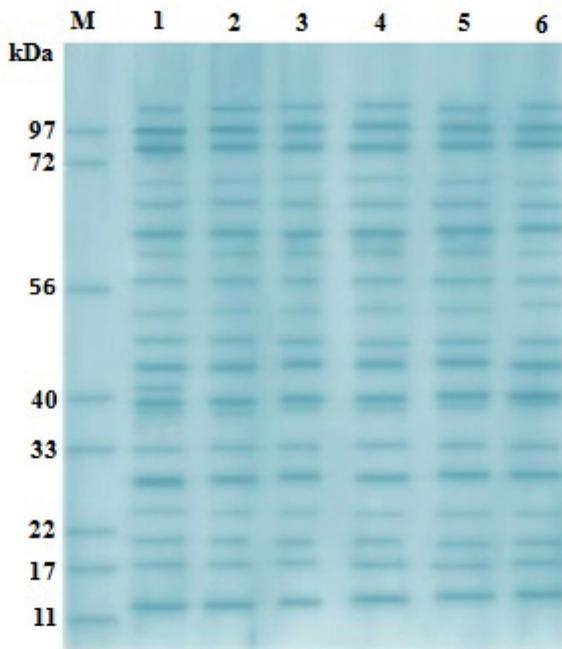


Fig. 3. Comassie Brilliant Blue stained protein profile of whole worm extract of *Fasciola gigantica* from different sheep livers (lane L1-L6), molecular weight marker (M).

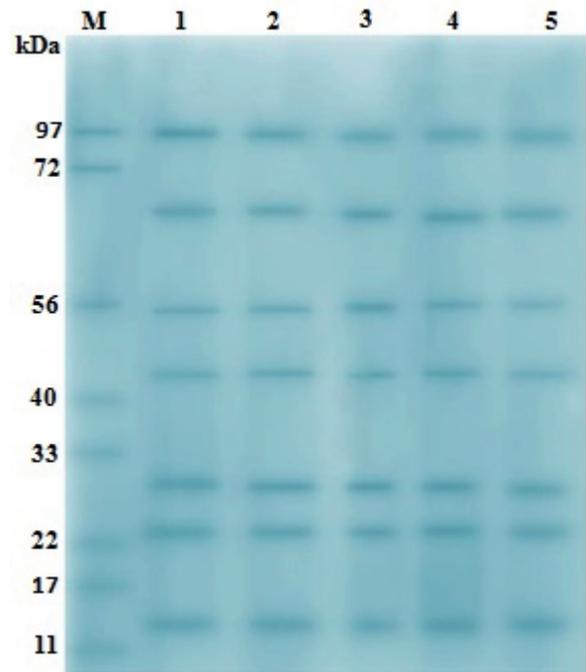


Fig. 4. Comassie Brilliant Blue stained profile of Excretory/ Secretory antigens of *Fasciola gigantica* from different sheep livers (lane L1-L5), molecular weight marker (M).

ously described *F. gigantica* isolates from different countries while the current studied isolates of *F. hepatica* formed a clade with *F. hepatica* reported from different countries. *Fascioides magma* (EF534995) was used as outgroup taxa and bootstrap values are shown next to clades (Fig. 2). By comparing the nucleotide sequence of ITS-2, the two species of *Fasciola* were found to differ at six nucleotide sites viz: 210, 234, 273, 279, 330 and 337. These include four transitions (CT) at 210, 234, 273 and 279; one indel at 330; and one transition (AG) at 337. The nucleotide compositions of *F. gigantica* and *F. hepatica* current isolates and other reported from other countries are summed in Table 1 and Table 2. The distance matrix analysis carried by p-distance and maximum composite likelihood method of MEGA 7 showed that the present *F. gigantica* isolates showed 100 % similarity and zero total character difference with already described species of *F. gigantica*, as far as their ITS-2 rDNA sequences are concerned while with *F. hepatica*, 5 – 6 bp differences were recorded. On the contrary, *F. gigantica* isolates showed maximum similitude (100 % similarity) and zero total character difference with already described species of *F. hepatica*; however, with *F. gigantica* 5 – 6 bp differences were noticed (Table 3).

SDS-PAGE and Immunoblotting

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE, 6 % stacking and 12 % resolving gel) protein profile of *F. gigantica* crude extract under reducing conditions, revealed 20 bands with molecular weight ranging from 14 – 110 kDa (Fig. 3).

Similarly Electrophoretic protein profile of total E/S antigen under reducing conditions revealed 7 polypeptide bands in the range of 14 – 97 kDa. These bands were detected at 14, 23, 27, 44, 54, 66 and 97 kDa (Fig. 4).

Western blotting of the crude somatic protein profile of *F. gigantica* with the sera of positive animals showed eight immunoreactive bands with molecular weights of 14, 23, 27, 38, 44, 66, 84 and 97 kDa (Fig. 5). Out of the 12 sera from *F. gigantica* infected sheep tested, 38 and 44 kDa proteins were found to give a consistent reaction with all the 12 sera samples and 84 kDa band was detected by 10 sera samples while as, the protein bands of 14 kDa was detected by 7, 23 and 27 kDa by 8, and 66 and 97 kDa by 9 sera samples. Further the cross reactivity of the somatic antigens of *F. gigantica* was determined by immunoblotting with six sera samples from sheep infected with paramphistomosis. With six sera samples from sheep infected with paramphistomosis, 38 and 44 kDa proteins bands were not detected by the any sera, the protein bands of 23, 27, 66, 84 kDa and 97 kDa were found to cross react with the 5, 3, 4, 1 and 4 sera samples of paramphistomosis infected sera respectively. 66 and 97 kDa proteins were even detected with the 2 sera samples out of 6 negative sera tested. The diagnostic sensitivity, specificity, accuracy and predictive values for somatic immunogenic proteins are given in Table 4. Therefore from the data, we conclude that 38 and 44 kDa proteins are quite specific for *F. gigantica* in crude antigen

Six immunodominant bands with molecular weights of 23, 27, 29, 33, 44 and 54 kDa were revealed by blotting the E/S fraction

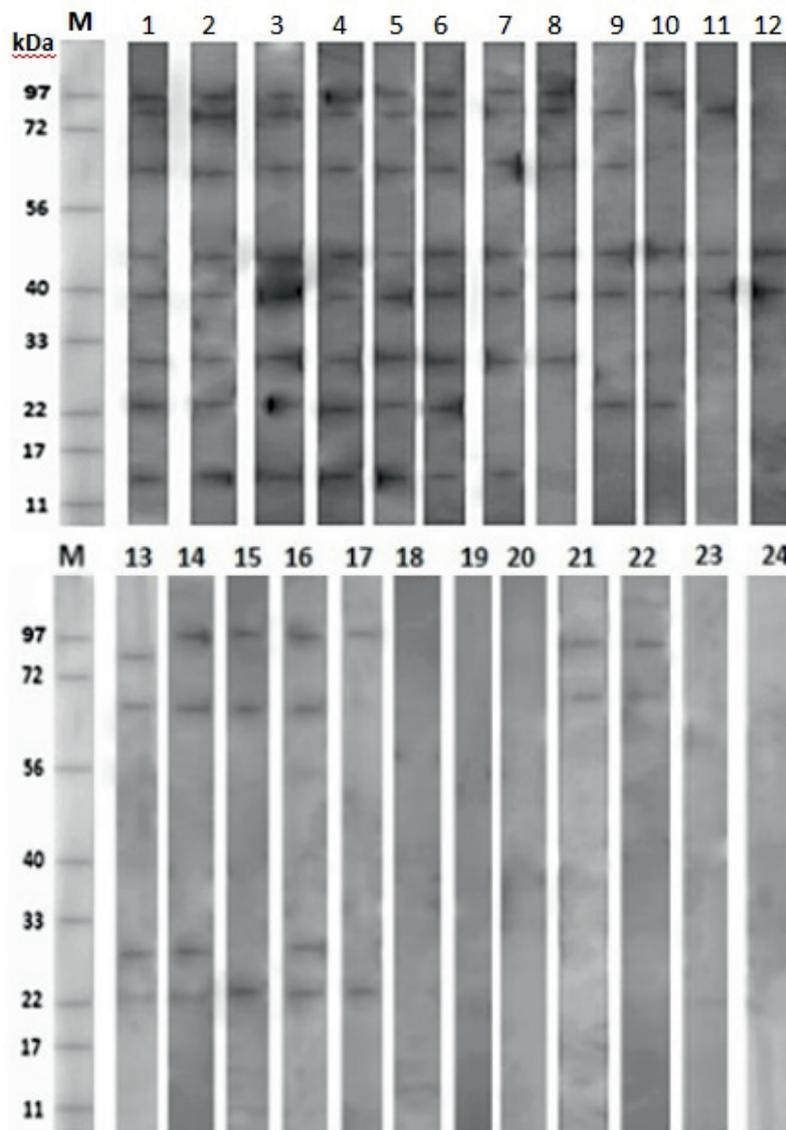


Fig. 5. Western blot analysis of somatic antigens of *Fasciola gigantica* against sera samples M: Molecular weight marker, 1-12: Positive sera of *Fasciola gigantica*, 13-18: Positive sera of *Paramphistomum* sp., 19-24: Negative control sera.

of proteins with the 12 sera samples of infected sheep with clinically diagnosed and parasitologically confirmed fasciolosis (Fig. 6). The protein bands of 27 and 33 kDa were found to give a consistent reaction with all 12 positive sera samples tested. Other protein bands with M.W of 23, 29, 54 and 44 kDa were detected only in 8, 10, 7 and 9 sera samples respectively out of total 12 positive sera samples. Moreover the bands of 23, 33 and 54 kDa were found to cross react with 3, 2 and 4 respectively out of 6 sera of paramphistomosis infected sheep. No band was observed when immunoblotted with 6 uninfected negative control sera. The diagnostic sensitivity, specificity, accuracy and predictive values for E/S immunogenic proteins are given in Table 5. Thus we conclude that the protein bands of 27 and 33 kDa are quite specific for *F. gigantica* in E/S antigen with high diagnostic specificity and sensitivity.

Discussion

The parasitic burden of trematode parasites has been a great challenge for veterinary practitioners in this part of world, and therefore it was felt to investigate one of the most economically important liver parasite i.e *Fasciola gigantica*. The overarching aims of the present investigation were to characterize this economically important parasite morphologically and molecularly and to identify its specific somatic and E/S antigens through Immunoblotting.

The use of ITS-2 gene in the present study for the species identification of liver flukes has been confirmed from a number of previous findings throughout the world (Adlard *et al.*, 1993; Ali *et al.*, 2008). Further the nucleotide sequences from mitochondrial DNA (NDI and COI) in addition to ITS-1 and ITS-2 genes of ribosomal

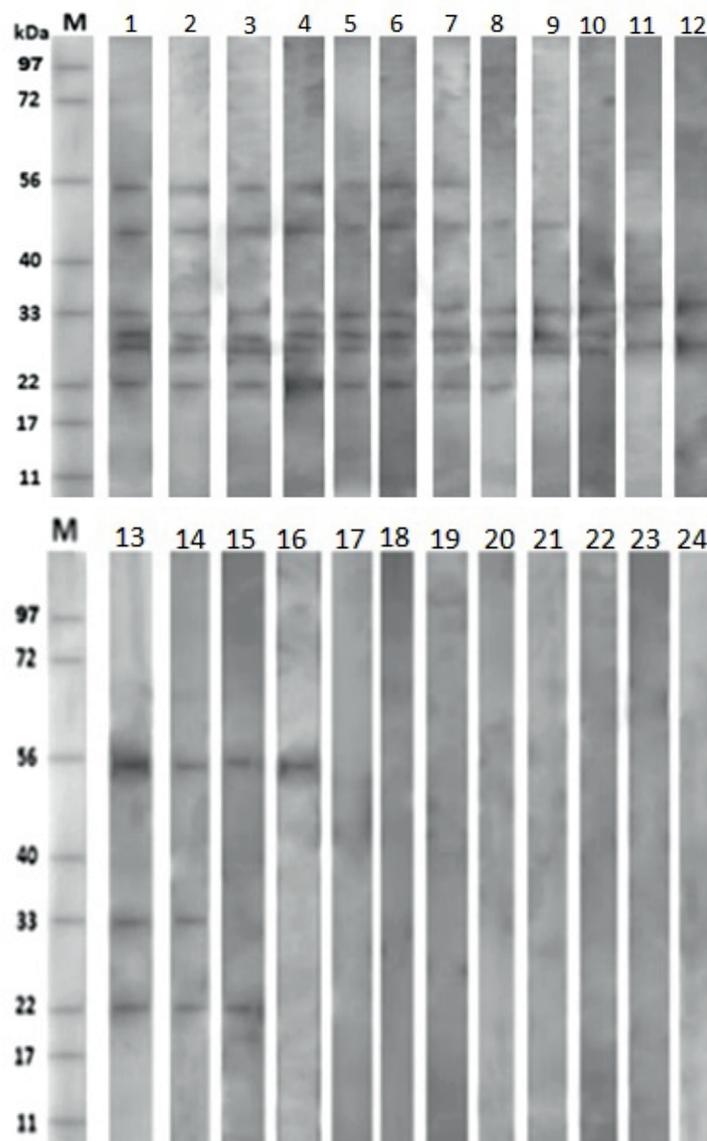


Fig. 6. Western blot analysis of Excretory/secretory antigens of *Fasciola gigantica* against sera samples M: Molecular weight marker, 1-12: Positive sera of *Fasciola gigantica*, 13-18: Positive sera of *Paramphistomum sp.*, 19-24: Negative control sera.

DNA have been effectively used for proper identification and differentiation of fasciolids as well as elucidating their origin and source of infection (Moghaddam *et al.*, 2004; Amor *et al.*, 2011; Itagaki *et al.*, 2005b). By comparing the nucleotide sequence of ITS-2, the two species of *Fasciola* were found to differ at six nucleotide sites *viz*; 210, 234, 273, 279, 330 and 337. These include four transitions (CT) at 210, 234, 273 and 279; one indel at 330; and one transition (AG) at 337. These results were in accordance with the studies of Adlard *et al.*, 1993, Agatsuma *et al.*, 2000, Huang *et al.*, 2004, Ali *et al.*, 2008 and Raina *et al.*, 2015, who also revealed six nucleotide differences in the ITS-2 between *F. hepatica* and *F. gigantica*. However, differences in seven nucleotide sites of ITS-2 between the two species of *Fasciola* have been reported

by Choe *et al.*, 2011 and Shafei *et al.*, 2014. Similar type of study was carried out by Galavani *et al.*, 2016 from West Azerbaijan Province Iran, who reported 100 % similarity of 1081 bp fragment of ITS-1 (428 bp), 5.8S (158 bp) and ITS-2 (366 bp) of their study isolates with *F. hepatica*. In their study, Only *F. hepatica* species was found to be distributed among sheep and cattle in West Azerbaijan Province Iran as the nucleotide sequence divergence for ITS2 among the isolates was found to be negligible or nil. The molecular characterization of *Fasciola* species collected from sheep in the present study confirmed the findings of Sharma *et al.*, 1989, who reported the presence of both *F. hepatica* and *F. gigantica* from the sheep of Kashmir valley. The phylogentic analysis of *Fasciola* species based on their ITS-2 sequences using Mini-

Table 4. Sensitivity, specificity, accuracy, Positive and negative predictive values of most prevalent positive bands using *Fasciola gigantica* somatic antigens

Test band (kDa)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Predictive Value	
				Positive (%)	Negative (%)
14	58.33	100	79.16	100	70.59
23	66.67	58.33	62.5	61.54	63.64
27	66.67	75	70.83	72.73	69.23
38	100	100	100	100	100
44	100	100	100	100	100
66	75	50	62.5	60	66.67
84	83.33	91.67	87.5	90.91	84.62
97	75	66.67	62.5	69.23	72.73

Maximum Evolution method showed that the seven isolates, JF1-JF3, JF9-JF12 formed the clade with *Fasciola gigantica* isolates from different country while five isolates JF4-JF8 formed the clade with *Fasciola hepatica* isolates from different country. ITS-2 sequence of *Fasciola gigantica* isolates in our study formed a single cluster with the *Fasciola gigantica* isolates from India, Indonesia and Japan with 100 % similarity. However the sequences obtained from GenBank from Kenya, Zambia, Iran and Burkina Faso got separately clustered. Similarly the ITS-2 sequence of *Fasciola hepatica* in our study formed a single cluster with the *Fasciola hepatica* isolates from Australia, Egypt, Turkey, Iran and Japan with 100 % similarity. Similar type of study by Prasad et al., 2008 showed close relationship of *Fasciola gigantica* isolates from Assam India with the *Fasciola gigantica* isolates from China, Indonesia, Japan, Egypt and Zambia with significant bootstrap values.

The present study was also focused to identify the somatic and E/S antigens of *F. gigantica* which can be used for immunodiagnostic purpose. In our studies with *F. gigantica*, protein profiling of crude somatic antigens by SDS-PAGE revealed 20 bands with

M.Ws ranging from 14 – 110. Similar studies were carried out on this fluke by number of authors who revealed different band patterns in their somatic extracts, however most of the distinct bands were found to be in the similar ranges. The study carried out by Gupta et al., (2003) revealed six polypeptide bands between the molecular weights of 27.7 – 37.5 kDa, while Meshgi et al., (2008) revealed 11 major bands having range between 18 – 68 kDa for somatic antigens of *F. gigantica*. In similar kind of study, Allam et al., (2002) reported 5 bands between 27 – 57.6 kDa, Gonec et al., (2004) reported 7 bands between 6.5 – 205 kDa, Upadhyay and Kumar (2002) reported 7 bands between 16 – 62 kDa, Hassan et al., (2014) reported 15 bands between 14 – 165 kDa. Along the similar lines, Sobhon et al., (1996) by SDS-PAGE profiling of the homogenized whole body of *F. gigantica* analyzed approximately 21 detectable bands with molecular weights ranging from 17 – 110 kDa, while as the tegument antigens (extracted by Triton X-100) electrophoresed only into eleven bands with M.Ws of 97, 86, 66, 64, 58, 54, 47, 38, 35, 19, and 17 kDa. Yokanath et al., (2005) by electrophoretic separation of somatic antigens of *F. gigantica*

Table 5. Sensitivity, specificity, accuracy, Positive and negative predictive values of most prevalent positive bands using *Fasciola gigantica* E/S antigens.

Test band (kDa)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Predictive Value	
				Positive (%)	Negative (%)
23	66.67	75	70.83	72.73	69.23
27	100	100	100	100	100
29	83.33	100	91.66	100	85.71
33	100	83.33	91.66	85.71	100
44	75	100	87.5	100	80
54	83.83	66.67	75	71.43	80

revealed 20 protein bands with molecular weights ranging from 14 – 156 kDa. Maleewong *et al.*, (1997) while working on human fasciolosis, revealed 22 polypeptide bands with molecular weight ranging from 14.4 to 94 kDa in the somatic extract of *F. gigantica* by SDS-PAGE. The number and molecular weight range of protein profile of whole worm extract of *F. gigantica* in our study was similar to the closely related species. For example, the study carried out by Cervi *et al.*, 1992 in case of *F. hepatica* revealed the somatic protein band pattern within the range of 14 – 94 kDa.

Protein profiling of E/S fraction of *F. gigantica* by SDS-PAGE revealed 7 polypeptide bands in the range of 14 – 97 kDa. These bands were detected at 14, 23, 27, 44, 54, 66 and 97 kDa. By SDS-PAGE analysis, Latchumikanthan *et al.*, (2012) reported 7 protein bands with molecular weights of 23, 25, 28, 43, 47, 52 and 66 kDa for total E/S antigen of *F. gigantica* which is similar to no. of bands in our results though the M.Ws of bands differs. The bands of 27, 44 and 54 kDa in our results may correspond to 28, 43 and 52 kDa bands in their study. The study carried out by Intapan *et al.*, (1998) on the protein profiling of E/S product of *F. gigantica* by SDS-PAGE analysis revealed six bands in the with the molecular weight ranging from less than 14.4 to 65 kDa, the band patterns of most of these bands were similar to our study. Along the similar lines, Goreish *et al.*, (2008) in their studies on protein profiling of somatic and E/S antigens of *F. gigantica* revealed that E/S antigens resolved in fewer bands compared to somatic extracts which is in line with our studies.

Immunoblotting test of resolved somatic bands with the sera of positive sheep for fasciolosis revealed the unique protein bands of 38 and 44 kDa, that were found to be quite specific for *F. gigantica* in crude antigen, as they were found to give consistent reaction with all the sera samples of positive sheep and were not cross reacting with sera of *Paramphistomum* infected and negative control sheep. Thus the protein bands of 38 and 44 kDa somatic antigens can serve as potential candidates for immunodiagnosis for fasciolosis. The antigenic band of 44 kDa was unique in our study, which was not found in early studies and needs further characterization to evaluate its immunodiagnostic potential. Similar study carried out by Maleewong *et al.*, (1997) on human fasciolosis revealed 13 bands with molecular bands ranging from less than 14.4 kDa to more than 94 kDa by immunoblotting analysis with the sera of positive patients for fasciolosis. One antigenic component of 38 kDa was found to give consistent reaction with the sera of all positive patients. Our study was partially in agreement with Maleewong *et al.*, (1997) in that we also found 38 kDa along with 44 kDa protein bands quite specific for *F. gigantica* in somatic antigen. However the study carried out by Yokananth *et al.*, (2005) showed the bands of molecular weight 28 and 34 kDa in crude somatic antigens of *F. gigantica* were recognized by the sera of positive animals and were considered highly specific immunodiagnostic antigens, which in our studies were found to be less specific antigens. Similar studies on *F. hepatica* by Gonenc *et al.*, (2004) showed protein bands in the molecular weight range of 24, 33, 35,

44 – 55, and 66 kDa by immunoblotting with the sera of positive sheep for *F. hepatica*. Further the studies carried out by Santiago *et al.*, (1986) in case of *F. hepatica* reported that the sera from experimentally infected rabbits recognized the major somatic antigens at 23–28 kDa and 33, 39, 52, 58, 84 and 120 kDa, While Santiago and Hillyer, (1988) reported the major somatic antigenic bands at 69, 64 and 56 kDa by Immunoblotting of somatic antigens with the sera of positive sheep and cattle.

In our study with the Excretory/Secretory protein fraction of *F. gigantica*, 6 immunodominant bands with molecular weights of 23, 27, 29, 33, 44 and 54 kDa were revealed by immunoblotting, among these the protein bands of 27 and 33 kDa were found to give a consistent reaction with all sera samples and may be considered quite specific for *F. gigantica* in E/S antigen with high diagnostic specificity and sensitivity. Thus the protein bands of 27 and 33 kDa E/S antigens can serve as potential candidates for immunodiagnosis for fasciolosis. Our study related to revelation of 27 and 33 kDa specific bands in the E/S antigens of *F. gigantica* support and augment the findings of Intapan *et al.*, (1998), who reported the prominent antigenic band of 27 kDa, was able to react consistently with sera of most positive patients with fasciolosis. In the study carried out by of Khabisi *et al.*, (2016), the polyclonal antibodies raised in rabbit against *F. hepatica* and *F. gigantica* E/S antigen reacted with five protein bands with molecular weights of 25, 27, 29, 62 and 67 kDa. By using a specific rabbit antiserum or the sera from cattle naturally infected with fasciolosis against the E/S products of *F. gigantica*, Attallah *et al.*, (2002) revealed the presence of a highly reactive antigen with molecular weight of 26 – 28 kDa. Similarly, Intapan *et al.*, (2003) observed 100 % sensitivity and 97.4 % specificity of 27 kDa E/S antigen of *F. gigantica* in the detection of human fasciolosis. Similar antigenic band patterns were also reported from the E/S protein fractions of *F. hepatica*. For example, by using enzyme linked immunotransfer blot probed by sera from *F. hepatica* infected horses and pigs, Gorman *et al.*, (1997) reported the immunoreactive bands at 14 – 19, 22 – 30, 35 – 37 and 40 – 42 in E/S antigens. According to their studies, the protein bands in the range of 22–33 kDa could be the potential candidates for immunodiagnosis of fasciolosis in horses and pigs. Similar studies carried out by Sampaio-Silva *et al.*, (1996) in case of human fasciolosis revealed that the protein bands of 25 and 27 kDa are highly specific for the E/S antigens of *F. hepatica* and could have high immunodiagnostic potential.

A number of other studies also detected a 27 kDa protein band as an immunodominant band of both somatic and E/S of *Fasciola* species (Santiago and Hillyer 1988; Attallah *et al.*, 2002; Dixit *et al.*, 2008; Kamel *et al.*, 2013). However the studies of Sampaio Silva *et al.*, (1996), Hammami *et al.*, (1997) and Rokni *et al.*, (2004), revealed the protein band of 29 kDa molecular weight as the principle band of the E/S antigen of *Fasciola* species for the serodiagnosis of fasciolosis. This 29 kDa protein band may correspond to cysteine-L-proteinase which was isolated and identified by Dalton *et al.*, (1996) and separated from the E/S products of *F. he-*

patica. Furthermore, certain important enzymatic components, like haemoglobinase (Coles and Rubano, 1988), glutathione-S-transferase (Hillyer *et al.*, 1992) and cysteine-L-proteinases of *F. hepatica* (Simth *et al.*, 1993; Dowd *et al.*, 1994) have been identified within the molecular weight range of 27.5 to 29 kDa in E/S products of the parasite. The Cysteine proteinases in the E/S products are recognized as significant proteins which are known to play role in the biological and immune-modulatory functions in the juvenile and adult flukes, and hence has become the focus of research in last few decades. Moreover they are known to induce high protective immunity in experimentally infected cattle (Dalton *et al.*, 1996).

Excretory-secretory (E/S) antigens have proved to be more useful for immunodiagnostic purpose as well as for protection against future infections than the somatic proteins (Dalton, J. and Heffernan, M. 1989; Parkhouse *et al.*, 1987). It is because E/S antigens more commonly get in touch with the host's immune system than somatic antigens, because the parasite excretes the content of the intestine like cathepsins and other enzymes with cytolytic activities. These enzymes degrade tissues and facilitate the invasion and migration of the parasite, and induce a stronger humoral immune response.

Different workers have showed different number of protein bands by SDS-PAGE and variable number of immunodominant bands by western blotting which could be attributed to existence of genetic variability in different isolates from different host species, geographical variations, use of different extraction buffers and handling errors while homogenization and protein extraction process can be counted too. The other reason could be the presence of both endogenous and host derived proteins primarily during isolation of flukes from thawed livers as suggested by De Vera *et al.*, (2009). Moreover, the pooled extract of grouped flukes based on morphological characters may have included potential hybrid within the supposedly *F. hepatica* and *F. gigantica* samples. No matter the difference exists in the number of protein bands or molecular weights of somatic and E/S polypeptides of *Fasciola* spp., the findings of various researchers suggest existence of antigens with promising diagnostic value in human and animals.

Conclusions

The results of the present study revealed the significant nucleotide difference in the ITS-2 of ribosomal DNA between *F. hepatica* and *F. gigantica*. Moreover the results revealed some unique antigenic profiles in the somatic and E/S fractions of *F. gigantica*, which could serve as potential candidates for immunodiagnosis. These proteins however, need to be further characterized in order to ascertain their therapeutic potential as vaccine candidates. This study is also important as veterinarians have raised their concern with regard to plasticity in various economically important parasites due to altered environmental conditions, and as such there is need to study various parasite groups in different geographical regions.

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Conflict of Interest

Authors state no conflict of interest.

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