Detection of *Fasciola hepatica* and *Fasciola gigantica* common and uncommon antigens, using rabbit hyper immune serum raised against their excretory–secretory and somatic antigens

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Abstract Fasciolosis is an important neglected helminth disease caused by two liver flukes, *Fasciola hepatica* and *Fasciola gigantica*. The two species of *Fasciola* are usually different in their morphological and molecular features. They have also common and uncommon antigens in both their somatic and excretory secretory metabolites. In this study, we compared somatic and excretory–secretory (ES) antigens of *F. hepatica* and *F. gigantica*, by using rabbit hyper immune serum raised against these antigens. Adult worms were collected from bile ducts of infected animals and species of the fluke was confirmed by RFLP-PCR. ES and somatic antigens of both species were prepared. Rabbits were subcutaneously immunized with either ES or somatic antigens to produce antibodies against these antigens. SDS-PAGE pattern of *F. hepatica* and *F. gigantica* somatic antigens was similar and both of them revealed 30 protein bands, ranging from 18 to 180 kDa. In contrast, SDS-PAGE pattern of ES antigen of the two species was different. While protein bands with molecular weight of 18, 27, 29, 48, and 62 kDa were common in both species, bands of 19, 45, 55 and 58 kDa were only noticed in *F. hepatica* ES antigen. Rabbit polyclonal antibodies, raised against *F. hepatica* and *F. gigantica* ES antigen, reacted with main five protein bands, 25, 27, 29, 62 and 67 kDa and polyclonal antibodies raised against somatic antigens of both species reacted with three protein bands,

25, 27 and 72 kDa. Thus, the 25, 27 and 29 kDa protein bands may serve as immunodominant antigens, which might be considered for serodiagnosis of fasciolosis. Moreover, bands of 62 and 67 kDa in ES antigen and 72 kDa in somatic antigens of both species were immunodominant and might be suitable candidate for development of serological assays for diagnosis of fasciolosis.

Keywords Western blotting · *Fasciola* · Somatic antigen · Excretory–secretory antigen

Introduction

Fasciolosis is a worldwide neglected parasitic disease of domestic animals and human (Hotez et al. 2012). The number of human fasciolosis has been increasing in the last decades in a few countries and the disease is considered as an important human parasitic infection in several areas of the world, including Bolivia, Ecuador, Peru, Viet Nam, Egypt and Iran (Soliman 2008; Rokni 2008; Sarkari et al. 2012; WHO 2010). It has been estimated that about 17 million people are infected with *Fasciola* spp. around the world (Mas-Coma et al. 2009).

The causative agents of both human and animal fasciolosis are two main species of the fluke, *Fasciola hepatica* and *Fasciola gigantica*. *Fasciola gigantica* is present mainly in tropical and *F. hepatica* in temperate areas, while both species overlapped in subtropical areas including Egypt and Iran (Soliman 2008; Mas-Coma et al. 2009; Moghaddam et al. 2004; Shafiei et al. 2014).

The two species of *Fasciola* are usually different in morphological features, such as body length and width. However, because of variations in size of these two species and the presence of intermediate forms, morphometric

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features are not appropriate characters for distinguishing of these two species of *Fasciola*. Nowadays, molecular approaches have been considered as suitable method for discrimination of the *Fasciola* species (Shafiei et al. 2013; Shafiei et al. 2014).

These two species of fluke have common and uncommon antigens in both their somatic and ES metabolites. Study of Meshgi revealed that *F. hepatica* and *F. gigantica* have at least six common major peptide bands in their ES products with molecular weights of 15–24 kDa (Meshgi et al. 2008). It was also demonstrated that the two species have differences in somatic proteins where *F. gigantica* had 11 major protein bands with molecular weights of 18–68 kDa and *F. hepatica* had eight distinct bands with molecular weights of 18–62 kDa (Meshgi et al. 2008).

The common antigens of two species might be useful for diagnosis of human or animal fasciolosis when the species of the parasite is not a matter of concern, while the uncommon antigens which are different in each species, might be appropriate antigens for either diagnosis of fasciolosis, based on the species of the parasite, or for vaccine development. Different antigenic fractions of *Fasciola* have been used for serological diagnosis of human fasciolosis. The use of specific antigens, purified from somatic or ES products of *Fasciola* spp. in ELISA or immunoblotting system, yielded satisfactory sensitivity and specificity as reported in different studies (O'Neill et al. 1999; Rokni et al. 2003; Espinoza et al. 2007).

Among the subunit antigens, the Cathepsin-L, a component of *Fasciola* ES antigens, has received the most attention (O'Neill et al. 1999; Rokni et al. 2002). It is obvious that nature and quality of purified antigen contribute to the validity and value of serological diagnostic tests. In order to increase the sensitivity and specificity of fasciolosis diagnostic tests, isolation, purification and characterization of somatic and ES antigens of both species of *Fasciola* is necessary (Dalimi et al. 2004). The current study aimed to find out the common and uncommon antigens of *F. hepatica* and *F. gigantica*, using rabbit hyper immune serum raised against ES and somatic antigens of these two species of *Fasciola*.

Materials and methods

Sample collecting

Fasciola adult worms were obtained from bile ducts of infected animals (sheep and cattle) from local abattoirs in Shiraz, south of Iran. *F. hepatica* was isolated from sheep while *F. gigantica* was isolated from cattle. The worms were washed five times with PBS (pH 7.2) and transferred

into 70 % alcohol, for molecular assay, into PBS for somatic antigen preparation, and into RPMI 1640 medium for ES antigen preparation.

DNA extraction and PCR-RFLP

Molecular identification of *Fasciola* species was performed as previously described (Shafiei et al. 2014). Briefly, a part of the apical and lateral zone of the adult flukes were removed and homogenized. DNA was extracted from the homogenized materials using phenol–chloroform method. PCR was performed using ITS1 forward; 5'-TTGCGC TGATTACGTCCCTG-3 and reverse; 5'-TTGGCTGCGC TCTTCATCGAC-3' primers. PCR products were subjected to fast digestion with *RsaI* restriction enzyme. The restriction fragments were separated on 2 % agarose gel and visualized with ethidium bromide.

Preparation of somatic antigen

F. hepatica and *F. gigantica* adult worms were used for preparation of somatic antigens. Adult worms were homogenized in PBS, using a tissue grinder. A cocktail of anti-protease was used during antigen preparation. Following homogenization, the sample was centrifuged at 13,000g for 30 min at 4 °C, supernatant was removed and stored at –20 °C until use.

Preparation of excretory–secretory (ES) antigen

Intact flukes collected from the bile ducts of infected animals were washed five times with PBS. The worms were transferred into culture flask containing RPMI-1640 (one fluke per 1 ml), 100 IU of penicillin and 100 µg/ml of streptomycin, and incubated at 37 °C for 14–16 h. Afterward, worms were removed and the remaining media was centrifuged at 13,000g for 30 min. Supernatant was dialyzed against PBS, lyophilized and stored at –20 °C until future use.

Production of Polyclonal antibodies against somatic and ES antigen

Eight female rabbits, weighing 1.5–2 kg, were allocated in two groups and each four rabbits were subcutaneously immunized with either ES or somatic antigens. In the first injection, each rabbit received 500 µg of antigens, mixed with equal volume of Freund's complete adjuvant followed by three 7 days interval of injections with Freund's incomplete adjuvant. Blood samples were collected, 10 days after the last injection, and examined by ELISA to verify anti-*Fasciola* antibodies.

Enzyme linked immunosorbent assay (ELISA)

Micro plates (Nunc, Nalgene, Nunc International, Roskilde, Denmark) were coated with 5 µg/ml of *F. hepatica* or *F. gigantica* somatic or ES antigens in coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6) and incubated at 4 °C overnight. After five times washes by phosphate buffered saline–Tween 20 (PBST, pH 7.4 containing 0.05 % Tween 20), unbinding sites were blocked with 3 % skimmed milk in PBS/Tween. The plates were washed as before and rabbit serum, at dilution of 1/100, was added and incubated for 90 min at room temperature. The wells were washed as before, and 100 µl of horseradish peroxidase (HRPO)-conjugated anti-rabbit IgG (Sigma) at 1/4000 dilution in PBST was added, and plates were incubated for 60 min at RT. After washing as before, the plates were incubated with chromogen/substrate (100 µl/well of OPD, 0.025 % H₂O₂ in 0.1 M citrate buffer, pH 5). The absorbance at 490 nm was checked with an ELISA microplate reader.

SDS-PAGE and western blotting assay

F. hepatica and *F. gigantica* ES and somatic antigens were separated by SDS-PAGE and evaluated by rabbit hyper immune serum, raised against each antigen. SDS-PAGE was performed under reducing condition using 12 % separating gel and 5 % stacking gel in a Bio-Rad apparatus at 25 mA/gel for 1 h. Separated antigen was transferred to nitrocellulose membrane, and the membrane was embedded in 5 % skimmed milk, as blocking solution, for 2 h. The membrane was washed by washing buffer (10 mM Tris, 150 mM NaCl, and 0.05 % Tween 20; pH 7.4). Each of produced rabbit hyper immune serum [1/100 dilution in washing buffer with 1 % bovine serum albumin (BSA)], were applied to the membrane and incubated for 90 min. After three washes (10 min each), peroxidase conjugated anti-rabbit IgG (Sigma) at dilution of 1/4000 was added and incubated for 60 min at RT. After three washes, as before, the reaction between antigen and antibody was visualized by adding diaminobenzidine (DAB) substrate (0.1 % H₂O₂ + 1 mg/ml DAB in 50 mM Tris–HCl, pH 7.6).

Results

SDS-PAGE pattern of *F. hepatica* and *F. gigantica* somatic and ES antigens

SDS-PAGE pattern of *F. hepatica* and *F. gigantica* somatic antigens were somewhat similar where both antigens revealed 30 protein bands ranging from 18 to 180 kDa.

Intensities of some protein bands including 22.5, 27, 31 and 62 kDa in SDS-PAGE pattern of *F. gigantica* somatic antigen was higher than *F. hepatica* somatic antigen. In addition, intensities of 31 and 53 kDa protein bands of *F. hepatica* somatic antigens were strongly higher than those of *F. gigantica*. In both species 25, 37.5 and 41 kDa protein bands revealed high intensities in comparison to other protein bands (Fig. 1).

SDS-PAGE pattern of ES antigens of two species was different. Protein bands with molecular weights of 18, 27, 29, 48 and 62 kDa were common in both species whereas protein bands of 19, 45, 55 and 58 kDa were only detected in *F. hepatica* ES antigens. The number of protein bands of ES antigen in comparison to somatic antigen was less and all of nine protein bands of ES antigens (18, 19, 27, 29, 45, 48, 55, 58, and 62) were available in somatic antigens of both species.

Western blotting analysis of ES and somatic antigens

Polyclonal antibodies raised against *F. hepatica* somatic antigen detected 17 bands ranging from 22.5 to 180 kDa, whereas polyclonal antibodies to *F. hepatica* ES antigen detected 11 bands, ranging from 25 to 180 kDa in somatic antigen of *F. hepatica*. Polyclonal antibodies raised against *F. gigantica* somatic antigen detected 13 bands ranging from 25 to 135 kDa and polyclonal antibodies raised against *F. gigantica* ES antigen detected 11 bands ranging from 22.5 to 180 kDa in *F. hepatica* somatic antigen. All of produced polyclonal antibodies commonly detected bands of 25, 27, 62, 72, and 135 kDa in *F. hepatica* somatic antigen.

Fig. 1 SDS-PAGE pattern of *F. hepatica* and *F. gigantica* somatic and ES antigens stained with Coomassie Brilliant Blue stain. 1 and 2 *F. hepatica* ES antigen; 3 and 4 *F. gigantica* ES antigen; 5, 6 and 7 *F. hepatica* somatic antigen; 8 *F. gigantica* somatic antigen

Table 1 Detected protein bands in western blotting on *F. hepatica* ES antigen (h) and *F. gigantica* ES antigen (g) with rabbit polyclonal antibodies raised against *F. hepatica* somatic antigen, *F. gigantica* somatic antigen, *F. hepatica* ES antigen and *F. gigantica* ES antigen

Antigen molecular weight (kDa)	18		19		25		27		29		45		48		55		57		62		67		135	
Polyclonal antibodies against	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h
<i>F. hepatica</i> somatic antigen	–	–	–	–	+	+	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	+	+	–
<i>F. gigantica</i> somatic antigen	+	–	–	–	+	+	+	+	+	+	+	–	+	–	–	+	–	+	+	+	+	+	+	–
<i>F. hepatica</i> E/S antigen	–	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	+	+	+
<i>F. gigantica</i> E/S antigen	+	+	–	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	+	+	+	+	–

Antibodies raised against *F. gigantica* somatic antigen detected 19 bands with different molecular weight, varies from 20 to 180 kDa while antibodies raised against *F. hepatica* somatic antigen detected 15 bands with molecular weight of 20–180 kDa. Antibodies raised against *F. gigantica* ES antigen detected 21 and antibodies raised against *F. hepatica* ES antigen detected 16 bands in somatic antigen of *F. gigantica*. All of produced polyclonal antibodies commonly detected bands of 25, 27, 37.5, 39.5, 45, 48, 72 and 180 kDa in *F. gigantica* somatic antigens.

Antibodies raised against *F. hepatica* ES antigen detected six bands and antibodies raised against *F. gigantica* ES antigen detected seven bands in ES antigen of *F. hepatica*. Antibodies raised against *F. hepatica* and *F. gigantica* somatic antigens detected seven bands in ES antigen of *F. hepatica* ranging from 25 to 67 kDa. All of produced polyclonal antibodies commonly detected bands of 25, 27, 29, 62, and 67 in ES antigen of *F. hepatica*.

Antibodies raised against *F. gigantica* ES antigen detected nine different bands with molecular weight of 18–135 kDa, whereas antibodies raised against *F. hepatica* ES antigen detected six bands of 25–135 kDa. Antibodies to *F. gigantica* somatic antigen reacted with ten bands of 18–135 kDa while antibodies against *F. hepatica* somatic antigen reacted with eight bands of 25–135 kDa. All of produced polyclonal antibodies commonly reacted with bands of 25, 27, 29, 62, 67 and 135 kDa of *F. gigantica* ES antigen. Tables 1 and 2 show the details of different components of ES and somatic antigens of *F. hepatica* and *F. gigantica*, detected by rabbit antibodies, raised against these antigens.

Discussion

Different antigens of *Fasciola* including somatic, ES, recombinant and tegumental antigens have been used for immunodiagnosis of human or animal fasciolosis (Morales

and Espino 2012; Rokni et al. 2003; Shafiei et al. 2015). The present study was carried out to determine common antigens of *F. hepatica* and *F. gigantica* somatic and ES antigens, using rabbit polyclonal antibodies raised against these two sets of antigens.

Species specific or common antigens of *F. hepatica* and *F. gigantica* have been reported in different studies. Meshgi et al. (2008) showed the presence of 11 (ranging from 18 to 68 kDs) protein bands in somatic antigens of *F. gigantica* and 8 (ranging from 18 to 62 kDs) protein bands in somatic antigens of *F. hepatica*. Maleewong et al. (1997) and Velusamy et al. (2006) respectively reported 11 (ranging from 14 to 94 kDa) and 23 (ranging from 12 to 96 kDa) protein bands in SDS-PAGE of *F. gigantica* somatic antigens.

Allam et al. (2002) and Gonence and Sarimehmetoglu (2004) in two different studies reported fewer protein bands in SDS-PAGE pattern of *Fasciola* in comparison to our study. Study of Yokananth et al. (2005), about electrophoretic separation of *F. gigantica* somatic antigen, showed 20 protein bands (ranging from 14 to 156 kDa) (Yokananth et al. 2005). Eight protein bands reported in their study are common with those that we are seeing in the current study. De Almeida et al. (2007) reported 17 protein bands (ranging from 2 to 80 kDa) in *F. hepatica* somatic antigen (De Almeida et al. 2007).

Meshgi et al. (2008) reported six common protein bands (ranging from 15 to 42 kDa) for SDS PAGE pattern of both *F. hepatica* and *F. gigantica* ES antigens. In a study by Gonenc et al. (2004) 23 protein bands (ranging from 6.5 to 205 kDa) for *F. hepatica* ES antigen have been reported. De Almeida et al. reported 19 protein bands (ranging from 2 to 80 kDa) for *F. hepatica* vomiting antigen (ES antigen) (De Almeida et al. 2007). In the current study, five and nine protein bands have been respectively detected in *F. hepatica* and *F. gigantica* ES antigens. Differences in number and molecular weight of reported protein bands of ES or

Table 2 Detected protein bands in western blotting on *F. hepatica* somatic antigen (h) and *F. gigantica* somatic antigen (g) with rabbit polyclonal antibodies raised against *F. hepatica* somatic antigen, *F. gigantica* somatic antigen, *F. hepatica* ES antigen and *F. gigantica* ES antigen

Antigen molecular weight (kD)	20	22.5	25	27	31	32	33.5	35	37.5	39.5	41	43	45													
Polyclonal antibodies against	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h										
<i>F. hepaticas</i> somatic antigen	+	-	-	+	+	+	+	+	-	-	-	+	-	-	-	+	+									
<i>F. gigantica</i> somatic antigen	+	-	+	-	+	+	+	+	-	+	-	+	-	-	+	+	-									
<i>F. hepatica</i> E/S antigen	-	-	-	-	+	+	+	+	+	+	-	-	+	-	+	+	-									
<i>F. gigantica</i> E/S antigen	-	-	-	+	+	+	+	+	+	-	+	-	+	-	+	+	-									
Antigen molecular weight (kD)	48		51		53		55		57		62		67		72		75		90		110		135		180	
Polyclonal antibodies against	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h	g	h
<i>F. hepaticas</i> somatic antigen	+	+	-	+	-	+	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	-	+	+	+	+
<i>F. gigantica</i> somatic antigen	+	-	+	-	+	-	-	-	+	+	-	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-
<i>F. hepatica</i> E/S antigen	+	-	+	-	-	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+	+	+
<i>F. gigantica</i> E/S antigen	+	-	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	-	+	+	+

somatic antigens of *Fasciola* spp. in various studies might be related to differences in the adult worms which have been isolated from different hosts.

In study of Gonence and Sarimehmetoglu (2004) serum of infected sheep, with *F. hepatica*, reacted with protein bands of 24, 33, 35, 44–55, and 66 kDa of *F. hepatica* somatic antigen, and protein bands of 24, 33, 39.5, 42 and 44–55 kDa of *F. hepatica* ES antigen. In other study, serum of human infected with *F. hepatica* reacted with bands of 8, 12, 15, and 24 kDa in western blotting on *F. hepatica* vomiting antigen (De Almeida et al. 2007). In our study, rabbit polyclonal antibodies to *F. hepatica* ES antigen detected 11 bands in *F. hepatica* somatic and ES antigen, most of them are different from those reported by De Almeida et al. (2007).

It has been shown that sera of cattle, sheep and buffalo, infected with *F. gigantica*, reacted respectively with 5, 8 and 11 protein bands of *F. gigantica* somatic antigen (Yokanant et al. 2005). In the present study rabbit polyclonal antibodies raised against *F. gigantica* somatic and ES antigen respectively reacted with 19 and 21 protein bands of *F. gigantica* somatic antigen. Source of infection (natural or experimental infection) might contribute to the detection of different protein bands in somatic or ES antigens of adult worm. In natural infection, hosts are temporary exposed to ES antigens but this does not happen in experimental infection or in immunized hosts.

Several studies pointed out that a 27 kDa protein band is an immunodominant protein band of ES and somatic antigens of *Fasciola* (Santiago and Hillyer 1988; Attallah et al. 2002; Dixit et al. 2008; Kamel et al. 2013; Intapan et al. 1998). However, in few studies, a 29 kDa protein band has been suggested as a principle protein band of *Fasciola* ES antigen for diagnosis of fasciolosis (Sampaio Silva et al. 1996; Hammami et al. 1997; Rokni et al. 2004). In our study, all of produced polyclonal antibodies, against ES and somatic antigens of *F. hepatica* and *F. gigantica*, commonly reacted with the 27 kDa protein band in western blotting on somatic or ES antigen of both species of *Fasciola*. In addition, a 25-kDa protein band was commonly detected in both ES and somatic antigens by all of produced polyclonal antibodies.

Taken together, in the present study rabbit polyclonal antibodies, raised against *F. hepatica* and *F. gigantica* ES antigen reacted with main five protein bands and polyclonal antibodies raised against somatic antigens of both species reacted with three protein bands (25, 27 and 72 kDa). Accordingly, the 25, 27 and 29 kDa protein bands may serve as immunodominant antigens that can be considered for serodiagnosis of fasciolosis. Moreover, bands of 62 and 67 kDa in ES antigen and 72 kDa in somatic antigens can be considered as immunodominant antigens and might be suitable candidates for development of serological assays for diagnosis of fasciolosis.

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Compliance with ethical standards

Conflict of interest None.

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