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Comparative study on isoenzyme patterns of *Fasciola hepatica* and *Fasciola gigantica*

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Abstract. Differences in the number of isoenzymes and their electrophoretic mobility are very valuable tools for characterization of closely related species of a certain parasite. This study aimed to compare the isoenzymatic patterns of *Fasciola hepatica* and *Fasciola gigantica*.

Adult *F. hepatica* and *F. gigantica* were collected from infected livers of sheep and cattle and their species was determined by molecular method. Enzymes were extracted from the adult worms and subjected to electrophoresis through a polyacrylamide gel. The activities of nine enzymatic systems; including superoxide dismutase (SOD), glucose phosphate isomerase (GPI), glucose 6-phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH), malic enzyme (ME), nucleoside hydrolyse 1 (NH1), phosphoglucomutase (PGM), isocitrate dehydrogenase (ICD), and 6-phosphogluconate dehydrogenase (6PGD) were evaluated from both *Fasciola* species.

The enzymatic profile obtained for SOD system for *F. hepatica* were two-banded pattern whereas in *F. gigantica* it was a four-banded pattern. NH1 revealed one band in *F. hepatica* with relative mobility of 0.05 and two bands in *F. gigantica* with relative mobility of 0.05 and 0.66.

In ICD, GPI and G6PD enzyme systems, both *Fasciola* species revealed one band but with different relative mobility. Isoenzymatic profile of MDH, 6PGD, PGM and ME were the same in both species.

Findings of this study revealed that *F. hepatica* and *F. gigantica* have entirely different isoenzyme patterns in the enzymes of ICD, G6PD, GPI, PGM and SOD. These enzyme systems may be used for differentiation of these two species of *Fasciola*.

INTRODUCTION

Fascioliasis is a parasitic disease caused by two species of liver fluke, *Fasciola hepatica* and *Fasciola gigantica*. While *F. gigantica* is restricted to the Old World, *F. hepatica* has a very wide distribution and present in Asia, Africa, Oceania, Americas and Europe (Mas-coma *et al.*, 2009; Sarkari *et al.*, 2012). Fascioliasis is one of the most important helminthic infections of domestic livestock, in particular cattle and sheep, in tropical and subtropical areas of the world. Human fascioliasis has been reported from diverse geographical areas of the world. About 2.4–17 million people are currently infected with fascioliasis in 51 different countries from five continents, and with approximately 180 million people are at risk of infection (Mas-Coma *et al.*, 2014).

Morphological features, such as body length and width, have been traditionally used to differentiate the two species of *Fasciola*. However, variations in size, discrepancy of morphological features, and the presence of intermediate forms, making this approach unsuitable for differentiation of these two species (Valero *et al.*, 2001; Ashrafi *et al.*,

2006). Nevertheless, molecular methods can appropriately distinguish the two species using the sequencing of different genes including; internal transcribed spacer 1 (ITS1), ITS2, 28S rDNA, NADH dehydrogenase I (ND1) and cytochrome C oxidase I (CO1) genes (Mas-Coma *et al.*, 2009; Peng *et al.*, 2009; Shafiei *et al.*, 2013; Shafiei *et al.*, 2014).

Although molecular approaches remains as the most appropriate method for differentiation of these two species of *Fasciola*, an alternative method, might be the isoenzymatic analysis of these species. Isoenzymes are different molecular forms of the same enzyme that have different migration speeds in electrophoresis. Differences in the number of isoenzymes and their electrophoretic mobility along with quantitative differences are very valuable tools for characterization of closely related species or strains of a certain parasite (Leon *et al.*, 1989). This approach has been extensively used for characterization of *Leishmania* species isolated from human and animal reservoirs (Kallel *et al.*, 2008; Bin *et al.*, 2010; Hatam *et al.*, 2013). Isoenzyme characterizations have been used, by several researchers, to find out the intraspecies variation of parasitic helminthes by looking at differences in isoenzyme patterns of a gives strain or species of the worm. This approach has been more notably applied for characterization of *Schistosoma*, *Diphyllobothrium*, *Trichinella* and *Dicrocoelium* species and strains of *Echinococcus granulosus* (Agatsuma & Suzuki, 1983; Fukumoto *et al.*, 1987; Leon *et al.*, 1989; Turceková *et al.*, 2003). As an example, Turceková *et al.* (2003) reported that GPI and MDH enzymes are suitable for the discrimination of G7 and G1 of *Echinococcus granulosus*.

The current study was conducted to compare the isoenzymatic patterns of *F. hepatica* and *F. gigantica* and to detect possible differences in their enzyme patterns for possible differentiation of these two species.

MATERIAL AND METHODS

Sample collection

Fifteen sample of adult *F. hepatica* and ten samples of *F. gigantica* were collected from infected liver of sheep and cattle, obtained from the local slaughterhouse in Shiraz, Iran. The samples were washed in phosphate-buffered saline (PBS) several times to completely remove the host tissues. The flukes were transferred to Falcon tube, containing PBS and immediately transferred to -20°C. A few of the isolated worms were kept at -70°C in ethanol for molecular evaluation.

Determination of *Fasciola* species

A PCR-RFLP method was used to distinguish *F. hepatica* from *F. gigantica* species. Genomic DNA was extracted from a portion of the apical and lateral zone of adult flukes, not including the reproductive organs. DNA fragments were amplified by polymerase chain reaction. The PCR-RFLP, using ITS1 primers and *RsaI* enzyme, was performed as previously described (Shafiei *et al.*, 2014).

Enzyme extraction

The isolated worms in PBS were crushed by tissue grinder in ice bath. The solution was centrifuged at 3000 g for 20 min at 4°C. Supernatant was discarded and the remaining pellet was washed three times with PBS and centrifuged as before.

An equal volume of enzyme stabilizers (2 mM Dithiothreitol, aminocaproic-acid, EDTA) was added to the pellet and mixed thoroughly. The samples were subjected to seven cycles of freezing (at -191°C) and thawing (at 30°C). Samples were centrifuged at 18000 g for 70 min at 4°C and the obtained solution was divided into aliquots and stored at -70°C until use.

Enzyme electrophoresis

Enzyme electrophoresis was done using discontinuous polyacrylamide gel electrophoresis. Electrophoresis was carried out, using 4% stacking gel, 8% separating gel, and a stacking buffer composed of Tris (pH 6.8),

Table 1. Materials for visualizing of each enzyme in polyacrylamide gel

Enzyme	Buffer	Substrate	Coenzyme
SOD	NaH_2PO_4, Na_2HPO_4	Riboflavin	Not needed
GPI	Tris-HCl 0.3 mol pH=8	D- F6P	NADH
MDH	Tris-HCl 0.3 mol pH=8	Malic acid	NAD
ME	Tris-HCl 0.3 mol pH=7.4	Malic acid	NADP
NH	Tris-HCl 0.3 mol pH=7.4	Inosine	Not needed
PGM	Tris-HCl 0.3 mol pH=8	D-G1,6 diphosphate	NADP
ICD	Tris-HCl 0.3 mol pH=7	Isocitrate	NADP
6PGD	Tris-HCl 0.3 mol pH=7.4	6 phospho glucunate	NADP
G6PD	Tris-HCl 0.3 mol pH=7.4	G6P	NADP

a resolving buffer of Tris (pH 8.9), and a tank buffer of Tris glysin (pH 9). The gels were run for 45 min at 30 mA. Both *Fasciola* species were evaluated for the activities of nine enzymatic systems including; superoxide dismutase (SOD), glucose phosphate isomerase (GPI), glucose 6-phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH), malic enzyme (ME), nucleoside hydrolyse 1 (NH1), phosphoglucomutase (PGM), isocitrate dehydrogenase (ICD), and 6-phosphogluconate dehydrogenase (6PGD). Materials which have been used for visualizing of each isoenzyme, including substrates, coenzymes and catalysts buffers are listed in Table 1. After staining, the relative mobility of the isoenzyme band (RF) and isoenzyme banding patterns (IBP) of two *Fasciola* species were determined and compared to each other.

RESULTS

In this study activities of nine enzymatic systems including SOD, GPI, G6PD, MDH, ME, NH_1, PGM, ICD, and 6PGD were evaluated in *F. hepatica and F. gigantica.*

Isoenzymes of isocitrate dehydrogenase (ICD)

In ICD enzyme system, both *Fasciola* species showed one isoenzyme band but with different relative mobility. *F. hepatica* showed a band with relative mobility of 0.26 while the relative mobility of the isoenzyme band of *F. gigantica* was 0.21 (Fig. 1).

Isoenzymes of glucose 6-phosphate dehydrogenase (G6PD)

G6PD system revealed one band with relative mobility of 0.29 in *F. hepatica* and one band with relative mobility of 0.25 in *F. gigantica* (Fig. 2).

Isoenzymes of glucose phosphate isomerase (GPI)

GPI revealed one band with different relative mobility of 0.39 and 0.30 in *F. hepatica* and *F. gigantica* respectively (Fig. 3).

Isoenzymes of superoxide dismutase (SOD)

The enzymatic pattern of SOD was quite different in two species of *Fasciola*. The profile obtained for *F. hepatica* was a two-banded pattern with relative mobility of 0.25 and 0.55, whereas *F. gigantica* revealed a four-banded pattern with relative mobility of 0.27, 0.42, 0.55, and 0.65.

Isoenzymes of nucleoside hydrolyse 1 (NH1)

NH_1 revealed one band in *F. hepatica* with relative mobility of 0.05 and two bands in *F. gigantica* with relative mobility of 0.05 and 0.66, respectively.

Isoenzyme of malate dehydrogenase (MDH)

Isoenzymatic pattern of MHD of both *Fasciola* species were similar where they produced a three-banded pattern with relative mobility of 0.05, 0.12 and 0.21.

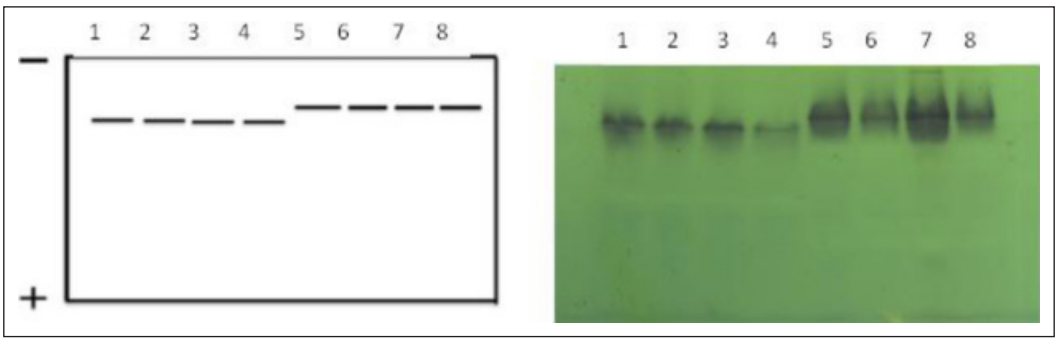

Figure 1. Electrophoretic profiles of ICD obtained with homogenate of *F. hepatica* (1-4) and *F. gigantica* (5-8).

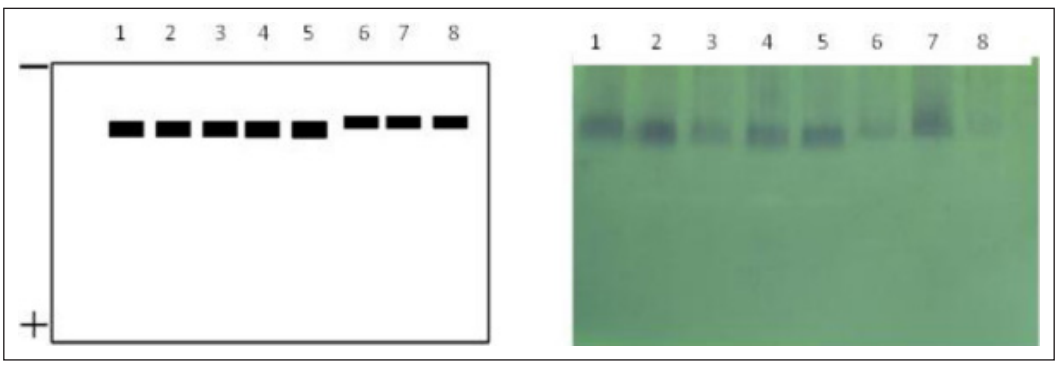

Figure 2. Electrophoretic profiles of G6PD obtained with homogenates of *F. hepatica* (1-5) and *F. gigantica* (6-8).

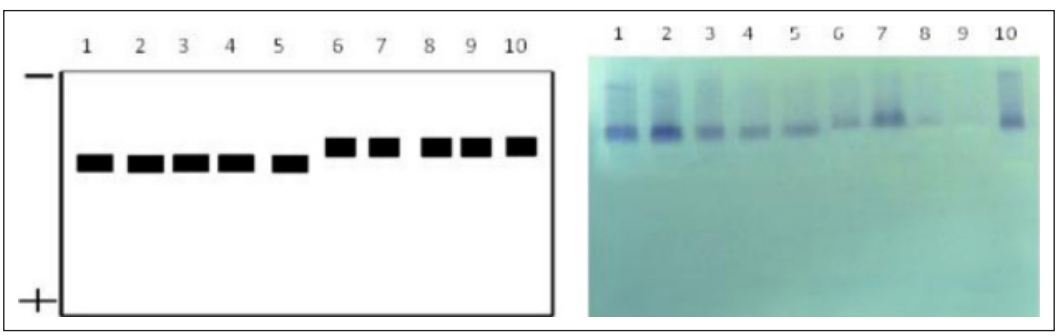

Figure 3. Electrophoretic profiles of GPI obtained with homogenates of *F. hepatica* (1-5) and *F. gigantica* (6-10).

Isoenzymes of 6-Phosphogluconate dehydrogenase (6PGD)

Isoenzymatic pattern of 6PGD was the same in both species where they produce a two-banded pattern with relative mobility of 0.16 and 0.02. In this enzymatic system, violet bands were seen in Tetrazolium staining. In addition to these bands, a few white bands were detected in the gel. These white bands were including the five bands for *F. hepatica* and two bands for *F. gigantica*.

Table 2. Isoenzymes pattern and relative mobility of *F. hepatica* and *F. gigantica* enzymes

Enzyme system	*Fasciola hepatica*		*Fasciola gigantica*	
	Number of band	Relative mobility	Number of band	Relative mobility
Isocitrate dehydrogenase (ICD)	1	0.26	1	0.21
Glucose 6-phosphate dehydrogenase (G6PD)	1	0.29	1	0.25
Superoxide dismutase (SOD)	2	0.55, 0.25	4	0.27, 0.42, 0.55, 0.65
Malate dehydrogenase (MDH)	3	0.21, 0.12, 0.05	3	0.21, 0.12, 0.05
Glucose phosphate isomerase (GPI)	1	0.39	1	0.30
6-Phosphogluconate dehydrogenase (6PGD)	2	0.16, 0.02	2	0.16, 0.02
Malic enzyme (ME)	3	0.18, 0.07, 0.05	3	0.18, 0.07, 0.05
Nucleoside hydrolyse 1 (NH1)	1	0.05	2	0.05, 0.66
Phosphoglucomutase (PGM)	2	0.1, 0.04	2	0.1, 0.04

Isoenzyme of phosphoglucomutase (PGM)

Isoenzymatic pattern of PGM was the same in both species where they produced two bands with relative mobility of 0.1 and 0.04.

Isoenzymes of malic enzyme (ME)

Isoenzymatic pattern of ME was the same in both species where they produce 3 bands with relative mobility of 0.05, 0.07 and 0.18.

Table 2 summarizes the enzyme pattern and relative mobility of the nine studied enzymes of *F. hepatica* and *F. gigantica*.

DISCUSSION

Fascioliasis is one of the most important helminthic infections of domestic livestock, in particular, cattle and sheep, and occasionally in humans in tropical and subtropical countries (Mas-Coma *et al.*, 2009; Hosseini *et al.*, 2015). The appropriate methods for differentiation of *F. hepatica* and *F. gigantica* are based on molecular approaches (Peng *et al.*, 2009; Shafiei *et al.*, 2013). Along with molecular methods, isoenzymatic analysis might be an applicable and suitable approach for differentiation of species of *Fasciola*. Differences in the number of isoenzymes are suitable features for characterization of closely related species or strains of a certain parasite (Leon *et al.*, 1989). A good example of in this regard detection of new zymodemes in *Leishmania* parasites which have been previously reported (Lemrani *et al.*, 2002; Ntais *et al.*, 2014).

Findings of the current study revealed that *F. hepatica* and *F. gigantica* have entirely different isoenzyme patterns in the enzymes of ICD, G6PD, GPI, PGM and SOD. The enzymatic profile obtained from SOD system in *F. hepatica* were a two-banded pattern whereas *F. gigantica* had two more bands. NH1 revealed only one band in *F. hepatica* while *F. gigantica* had one more band. Although in ICD, GPI and G6PD enzyme systems both *Fasciola* species revealed one band, but their relative mobility was quite different.

Leon-Ortega *et al.* (1988) evaluated two enzymes, MDH and ME, by discontinuous polyacrylamide gel in *F. hepatica*. In MDH system for *F. hepatica* three bands were reported and no differences were found in *Fasciola* which were isolated from different hosts, namely *Ovis aries* and *Capra hircus*. In our study, in MDH system three bands with relative mobility of 0.05, 0.12 and 0.21 were observed and no differences were seen between the two species. The numbers of bands in our study were similar to Leon-Ortega *et al.* (1988) study but the bands were

different in their location and width. The reason for these observations might be the location and region of sampling and also the host of the parasite.

Leon-Ortega *et al.* (1988) study on ME systems of *F. hepatica*, revealed three isoenzyme bands; one of them was near to the cathode. In contrast to MDH, in ME system the location of the band for the *Ovis aries* and *Capra hircus* were different. We observed three bands for ME enzyme for *F. gigantica* and *F. hepatica*. The relative mobility of the three bands was the same in both species. Comparing our findings with findings of Leon-Ortega study on ME system, the number of bands was the same and also was similar in width for the first band but with different width for the second band. For the third band, they were in the same location but the band was a bit thinner in our study.

Sanchez-Moreno *et al.* (1987) reported the pattern of SOD for *Fasciola* by polyacrylamide gel electrophoresis. In their study three bands with similar intensity were observed for the *F. hepatica*. We observed two bands for *F. hepatica* and three bands for *F. gigantica* in our study. The first and second bands were similar to those reported by Sanchez-Moreno but the third band was not seen in our study. Parasite's host and genotype might be contributed to these differences.

In our study, some nonspecific bands in white color were observed in few of the enzyme systems (e.g. 6PGD), while based on the method, violet color was expected. The white color was unusual but the white bands were still different in two species.

In the current study, among the five enzyme systems which had different activities if *F. hepatica* and *F. gigantica*, ICD might be considered as a superior system, since it well differentiated the two species, has a good activity, is cheaper than others and does not need linking enzyme.

Overall, findings of this study showed that the isoenzyme patterns of *F. hepatica* and *F. gigantica*, in at least five of the nine evaluated enzymatic systems, are different and these systems may well be used to distinguish the two species of *Fasciola* from each other. As a continuation of research on the issue, ccomparison of *Fasciola* isoenzyme patterns in more diverse hosts and evaluation of these patterns in intermediate form of *Fasciola* can be suggested.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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