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Original Article

Secretory Excretory and Somatic Immunogenic Antigens Profiles of Adult *Fasciola spp.*

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Abstract

Fascioliasis is a common human-animal disease that is reported in most parts of the world. Fascioliasis is also prevalent in different provinces of Iran. Since it has done no study on the excretory/secretory and somatic immunogenic antigens profiles of adult *Fasciola* in Iran, the present study was performed on the *Fasciola spp.* collected from Mazandaran province. For this purpose, the *Fasciola* worm was isolated from the liver of infected sheep, then its excretory/secretory and somatic antigens were prepared from adult worms. The protein of the samples was measured by the Lowry method. Then, somatic and secretory excretions were examined by SDS-PAGE and the protein profile of the two substances was determined. To evaluate the immunogenicity, the somatic and secretory excretions antigens of *Fasciola spp.* were injected into white rabbits and after boosting, the blood serum of the rabbits was collected and then Western blotting was performed on them and the results were evaluated. According to the results of Western blotting, 11 somatic antigen bands with a molecular weight of 149, 122, 99, 85, 75, 65, 50, 46, 40, 37, 30 kDa and 12 protein bands of excretory/secretory antigens with molecular weights of 100, 82, 75, 70, 58, 55, 47, 40, 38, 37, 30,25 kDa were observed in adult *Fasciola spp.* that immunogenic, which appear to have a protective effect or can be used to prepare a diagnostic kit.

Keywords: *Fasciola*, Protein profile, Secretory excretory, Somatic antigen

1. Introduction

Fascioliasis is one of the most common liver diseases in the world, which is caused by a trematode called *Fasciola spp.*, which belongs to the Fasciolidae family. Fascioliasis is one of the major causes of economic waste in the world's livestock, which besides herbivores, also affects many domestic and wild animals, as well as humans. During its life cycle, this parasite chooses two hosts (*Lymnae* snail as intermediate host) and herbivores (as main or final host). The parasite settles in the bile ducts of the liver of the main host. Its two pathogenic species, *Fasciola hepatica* and, *Fasciola gigantica*, are common in the world's livestock and Iran (1).

Compared to animal infections, human infection with *Fasciola spp.* are uncommon; however, human infection with *Fasciola* has been reported epidemically in over forty countries, including Europe, the Americas, Asia, Africa, the Western Atlantic, Australia, and Iran (2, 3). In Iran, besides the north of the country, the ecological conditions for the growth of *Fasciola* seem to be favorable in other places as well. According to a

report, over 23% of the sheep slaughtered in the country's slaughterhouses are infected with parasites (4).

In rat, both cellular and humoral immunity play a role in protecting this animal against infection. Oxygen (O_2) and nitric oxide (N0) secreted from rat peritoneal leukocytes may play an important role in killing *Fasciola hepatica*. The mechanism of production of free radicals is probably dependent on antibodies. The ability of cytokines IFN2 and TNF to produce nonspecific free radicals is an antibody-dependent Eosinophils may play an important role in the resistance of rats to *Fasciola*, and may kill the parasite by increasing free radicals from one cytotoxicity to the other (5).

Immunity in cattle is based on the tissue reaction that slows down the movement of the parasite in secondary infection due to calcification and fibrosis. Immune cells surround the parasite and kill it (5).

Conversely, acquired immunity in sheep is very weak or not seen at all against *Fasciola hepatica*, and even after stimulation of the immune system, there is no noticeable change in the number of parasites, which is why the animal may die during human infection. IgG4 and IgG1 are major antibodies in human fascioliasis (5, 6).

Due to the importance of the subject, research on rapid diagnosis or preparation of the anti-*Fasciola* vaccine is always relevant. For many years, many attempts have been made to use parasite antigens to make the host resistant to challenge infection, but most of these attempts have failed. Vaccination of mice and rats with products secreted from parasites in the culture medium has been somewhat successful, but this success has not been confirmed by other researchers.

Given the growing need of people for healthy animal protein sources and the increasing health and economic damage of this parasite and the increasing sporadic and epidemic spread of *Fasciola*, it is obvious that any action to eradicate or prevent this parasite will be valuable to human societies. In this regard, the study of the protein and antigenic profile of *Fasciola* excretory-secretory and somatic antigens; may provide appropriate solutions for the production of diagnostic kits or vaccines against *Fasciola* infections. Although many studies have been done in this field to date, in our country, no specific study has been done in practice.

Therefore, the present study was designed and performed to determine the protein and antigenic profile of *Fasciola spp.* excretory-secretory and somatic antigens isolated from sheep.

2. Materials and Methods

2.1. Collection of Adult *Fasciola spp.* Worms

In order to prepare and collect samples of adult *Fasciola spp.* worms, we referred to the slaughterhouse of Chalous city (Mazandaran Province). After opening the bile ducts of the infected livers of sheep, the *Fasciola spp.* worms were removed and kept in PBS, then stored in a laboratory at 4°C in the laboratory.

2.2. Preparation of Excretory-Secretory Products

To prepare excretory-secretory product from adult *Fasciola spp.* worms, first 10 live adult *Fasciola* worms were washed with PBS (pH: 7.2) in several steps and then place separately in a closed petri dish and add some PBS. The petri dish was poured so that the worms could float. The containers were placed at 37.5 °C for 24 hours.

After this time, excretory-secretory products were removed from the petri dish and transferred to the sterile test tubes, and then centrifuged at 10,000 RPM for 15 minutes. After separating the supernatant, the produced sediment was frozen at a temperature of -22°C and the obtained samples were transferred to the Biochemistry laboratory of the Biotechnology Department of Razi Institute along with dry ice.

2.3. Homogeneous Preparation of Adult *Fasciola spp.*

Four adult *Fasciola spp.* worms were poured into the test tube and twice the volume of PBS solution was added to it, then the samples were poured into a plastic container filled with ice to make a uniform and homogeneous solution.

An electric homogenizer was used to homogenize the samples and extract protein from the tissue. After crushing and homogenizing the samples, the solution was centrifuged at 10000 RPM for 30 minutes at 4 °C.

2.4. Lowry Protein Assay

Lowry method was used for protein measurement and all tubes were read at 750 nm with a spectrophotometer and the OD of solutions was calculated.

2.5. SDS-PAGE Test

After preparation of the samples, SDS-PAGE (5-10%) was tested with samples of excretory-secretory and somatic antigens of *Fasciola spp.* by staining with coomassie blue and silver nitrate (7).

2.6. Immunization of Rabbits

2.6.1. First Stage of Immunization: Injection of Antigens with Complete Freund Adjuvant

The following steps were performed to inject the prepared antigens into rabbits.

For the first injection to prepare the antigen, mix 100 µl (containing 0.13 mg of protein) of a homogeneous solution prepared from adult *Fasciola* with 900 µl of sterile saline solution to 1 ml of solution with 1 ml of adjuvant. Freund was stirred several times until gelatinous and homogeneous, according to standard and standard methods for preparing antigen, and a homogeneous mixture of milky, white, and thick was obtained (7).

The injection was performed at 8 to 10 points subcutaneously in a rabbit. A site close to the neck was selected as the main site and injection with a larger volume of antigen was performed at that site and the rest of the injections were performed around it.

To prepare the antigen from the excretory material, 1 ml of the excretory-secretory product prepared from the adult *Fasciola* (containing 1.3 mg/ml protein) which were stored at -20 °C were taken. After melting and turning into a complete liquid state, mixed with 1ml of Freund's complete adjuvant according to standard common methods in antigen preparation until the emulsion becomes homogeneous and uniform and in the form of an elastic gelatin solution (8).

The antigen prepared from excretory-secretory antigens of adult *Fasciola spp.* was injected into the back of the animal's neck at the junction of the head and neck subcutaneously in 8 to 10 points with a small volume and with a larger volume at a central point at the junction of the head and neck.

2.6.2. Second Stage of Immunization: Injection of Antigens with Incomplete Adjuvant

To increase the level of immunity (booster dose), for excretory-secretory and somatic antigens of adult *Fasciola spp.*, 3 injections with incomplete adjuvant were performed. For this purpose, 100 µl of a homogeneous antigen prepared containing 0.13 mg/ml of somatic *Fasciola* antigen was mixed with 900 µl of saline and then 1 ml of incomplete adjuvant was used according to the usual antigen preparation methods. The mixture was thoroughly mixed to make a homogeneous, almost solid, gelatinous emulsion. This was also done with samples of secreted excretory antigen (ES antigen) (8).

At this stage, the injections were performed intramuscularly. These injections were performed on the muscles of the two arms and legs of the animal, with the muscles of the back arm in the arm and the thigh muscles in the legs at the injection site. The amount of injected material was mixed with 1ml of excretory-secretory products with 1ml of incomplete Freund adjuvant and after homogenization and preparation of homogeneity, it was injected intramuscularly in rabbits.

2.7. Blood Sampling from Rabbits

Due to the need for the serum of immunized rabbits for Western blotting, blood samples were taken from a rabbit ear vein using a sterile scalpel and xylol. Blood was collected in sterile tubes and refrigerated for 8-12 hours. After centrifugation of the samples at 6000 RPM, the serums were separated for 10 minutes. After separating the sera, they were stored at -20°C until the

experiment.

2.8. Western Blotting Test

The western blotting test was performed according to its own instructions. In this way, first, the antibodies were transferred from the SDS-PAGE gel to the cellulose nitrate paper, and then they were mixed with the blood serum of the rabbits that had been boosted in four stages, and after the bands were revealed, they were added to the RF table. Value is used to calculate the weight of protein bands.

2.9. Statistical Analysis

Statistical analyses were carried out using SPSS Version 21. Kolmogorov-Smirnov test was used to ensure data normalization.

3. Results

3.1. Lowry Protein Assay

In the Lowry Macro test, the amount of protein obtained from the somatic antigen of adult *Fasciola spp.* was 11.43 mg/ml and from the excretory/secretory product was 1.3 mg/ml.

3.2. SDS-PAGE

In the first stage, after preparing the samples and their protein measurement, the SDS-PAGE test was performed by diluting the samples, but due to the high protein concentration, unspecified bands were obtained. Then, in order to better identify the bands, the protein dilution of the sample was reduced and with dilution (125 µl/ml), the experiment was performed.

In the SDS-PAGE test with coomassie blue staining for the somatic product of adult *Fasciola spp.* 12 bands with molecular weights of 263, 185, 145, 95, 85, 70, 66, 55, 49, 40, 37, 26 kDa and for excretory-secretory antigens of adult *Fasciola spp.* 11 with a molecular weight of 145, 95, 70, 68, 66, 60, 55, 50, 49, 40, 37 kDa were observed relative to the marker using the table Rf. Value bands (Figure 1) (6, 7).

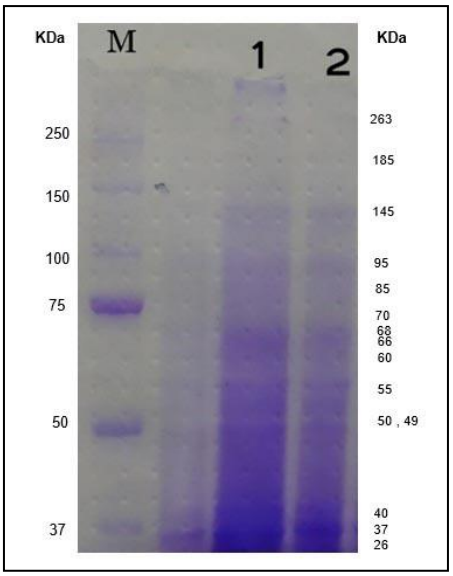

Figure 1. Bands obtained in SDS-PAGE test for excretory-secretory and somatic antigens of *Fasciola spp.*. M: Marker 1- Somatic protein of adult *Fasciola spp.* bands; 2- Protein bands of excretory-secretory product

3.3. Reaction of Animal to the Injection

In order to evaluate the immune system response and their reaction to the injected cases, 2 weeks after the first injection in rabbits, injection sites and positions were inspected.

In the case of injection of adult *Fasciola spp.* somatic antigens at the initial injection sites, prominent nodules and even wounds and cracks appeared that showed immune system stimulation. In the case of injection with adult *Fasciola spp.* somatic antigens with complete Freund adjuvant, at the injection site; large, distinct nodules with obvious sores and cracks were seen after 14 days. At the injection site with adult excretory-secretory product, some nodules were observed at the injection site with Freund's complete adjuvant behind the animal without scarring after 14 days.

3.4. Western Blotting

In the Western blotting test, with somatic antigen 11 specific bands with a molecular weight of 149, 122, 99, 85, 75, 65, 50, 46, 40, 37, 30 kD were obtained (Figure 2).

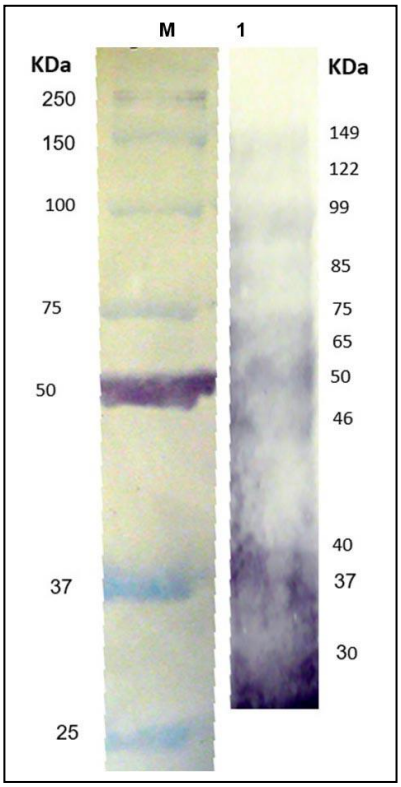

Figure 2. Bands obtained for adult *Fasciola spp*. antigen and immunized rabbit blood serum in the Western blotting test

In Western blotting test with the excratory-secretory products in comparison with marker, 12 protein bands with molecular weights of 100, 82, 75, 70, 58, 55, 47, 40, 38, 37, 30, 25 kDa was observed (Figure 3).

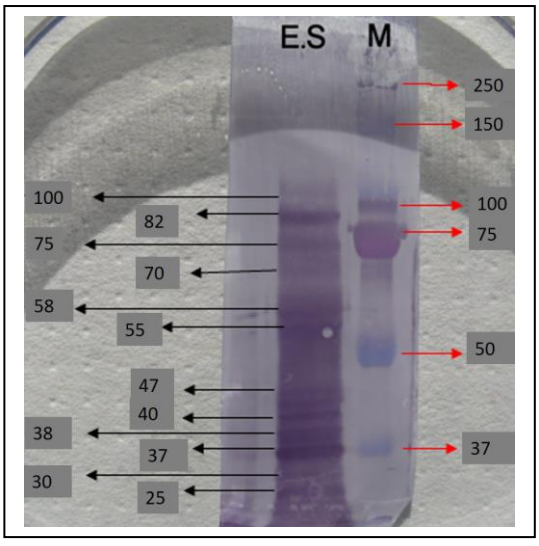

Figure 3. Bands obtained in Western blotting test for excretory-secretory products of adult *Fasciola spp*..

4. Discussion

In the present study, a number of *Fasciola spp.* worms from Mazandaran province (Chalous) were collected from native cattle. Collected parasites were detected as *Fasciola gigantica* in the laboratory. However, given the morphological and phenotypic variations of *Fasciola* worms, it can not be said with certainty that the collected *Fasciola* were exclusively *Fasciola gigantica*, because the intermediate forms of *Fasciola hepatica* and *Fasciola gigantica* are common in the region (4).

To develop a diagnostic kit or vaccine, it is necessary to know the components of the protein and antigen profile at different stages of the parasite's life cycle, such as adult worms, excretory-secretory antigens, and metacercariae. In the present study, after collecting the somatic and secretory-secretory antigens of adult worms, first, their protein content was measured and then in the SDS-PAGE test, the somatic protein profile was demonstrated 12-band with molecular weights of 263, 185, 145, 95, 85, 70, 66, 55, 49, 40, 37, 26 kDa. However for excretory-secretory antigens of adult *Fasciola spp.* 11 complete bands with molecular weights 145, 95, 70, 68, 66, 60, 55, 50, 37, 40, and 37 kDa were observed.

The debate over antiparasitic vaccines has become more serious since 1990. The increasing resistance of parasites to drugs, the toxicity of drugs to humans and animals, the resistance of snails to snailicide drugs, and the talk of environmental pollution and the form of control of intermediate hosts and parasite reservoirs have made the vaccine an effective and viable lever. With the advancement of biotechnology and molecular biology, new ideas were created, but in this way, many problems were encountered. According to some studies, vaccination of sheep with γ-irradiated metacercariae of *F. hepatica* affects the number and growth of the *Fasciola* population, leading to a reduction of liver damage during migration (9). Furthermore, the radiated metacercaria of *Fasciola gigantica* in cattle and sheep was reported to provide up

to 80% immunity (10). Although the immune system of cattle has reported that could kill *Fasciola gigantica* better than *Fasciola hepatica* and the results of the effectiveness of these vaccines were reported depending on factors such as the amount of radiation and the type of host (11).

Although attenuated parasite vaccines stimulate the host's immune system well, but due to lack of efficiency, they have received little attention. Therefore, most of the studies were directed toward preparing antigenic vaccines for helminthic diseases. *Haemonchus contortus* H11 antigen and GST in trematodes and cestodes are of this type. According to some studies in Iran, by using GST and saponin adjuvant, immunization against *Fasciola gigantica* was not very effective in sheep (12, 13). Result of another study indicated that vaccination of cattle with GST *Fasciola gigantica* also did not reduce the infection rate of the parasite (14). Contrary, in another study, the results of injecting GST intraperitoneally before a lethal dose of lipopolysaccharide gives the surviving 80% of C57BL/6 mice significantly reduced amounts of pro-inflammatory cytokines and chemokines, such as IL-1β, IL-12p70 and MIP-1α (15).

The other antigens that used include Fh12 in *Fasciola hepatica*, which is a group of proteins called FhSmIII that is common between *Fasciola hepatica* and *Schistosoma mansoni* (16). Vaccination with FhSmIII in cattle could reduce *Fasciola hepatica* infection by 55% (16), therefore, the use of GST and Fh12 has been recommended.

Cathepsin L is a protective antigen in *Fasciola* protease that is secreted in a culture medium in *Fasciola hepatica*. Vaccination with Cat-L1 showed a 53.7% reduction in infection, and the mixture of Cat-L1 and hemoglobin showed a 52% reduction in infection. Cat-L2 mixture with hemoglobin produced 72% protection in cattle, furthermore, more than 98% of the eggs expelled from the parasite did not have embryos. But no correlation was observed between protection and antibody production (11). In addition, cysteine proteases are able to inhibit penetrating the young *Fasciola* into the final host (sheep) intestine (6).

Some *Fasciola spp.* antigens have been used as a tool to detect fascioliasis in humans and animals. The presence of the cathepsin-like proteins 1L, 2L, B, and cysteine is involved in protecting and vaccinating the host. These enzymes also play an important role in the disease's diagnosis, even in the early stages. The cathepsin 1L antigens of adult *Fasciola gigantica* were evaluated by ELISA and concluded that it could be well used in the diagnosis of human and animal fascioliasis (16, 17). Two studies, using cathepsin L-like protease, reported 100% sensitivity and 97% specificity in diagnosing the infection in sheep as well as 100% sensitivity and specificity in humans by the ELISA method (18, 19). The 25 and 27 kDa proteins of *F.hepatica* has been reported that could be useful to identify human cases of fascioliasis between 92% and 98% (19). Also, the 30 kDa of somatic antigen has been used as a diagnostic antigen for the detection of fascioliasis infections (20). Furthermore, human and bovine fascioliasis was diagnosed with up to 96% using excretory-secretory antigen in the Dot-ELISA method, (21).

The result of a study indicated that the somatic protein profiles of *F. hepatica* and *F. gigantica* were similar and both of them showed 30 protein bands ranging from 18 to 180 kDa in SDS-PAGE. Contrary, the protein profiles of ES antigen of the two species were different. While the protein bands of 18, 27, 29, 48, and 62 kDa were shared in both species, but bands of 19, 45, 55 and 58 kDa were only identify in *F. hepatica* ES antigen. Result of the Western blotting indicated that *F. hepatica* and *F. gigantica* ES antigen, showed five protein bands, 25, 27, 29, 62 and 67 kDa and somatic antigens of both species showed, 25, 27 and 72 kDa (22). In the present study, according to the results of Western blotting, 11 somatic protein bands with a molecular weight of 149, 122, 99, 85, 75, 65, 50, 46, 40, 37, 30 kDa and 12 protein bands of excretory/secretory antigens with a molecular weight of

100, 82, 75, 70, 58, 55, 47, 40, 38, 37, 30, 25 KDa of adult *Fasciola spp.* were observed to appear to be immunogenic. They may have a protective effect on humans /animals or may be used to provide a suitable diagnostic kit.

With the advancement of molecular biology and biotechnology, it seems that control of fascioliasis by immunization is an achievable goal, but it requires various studies on *Fasciola* species because parasites, especially worms, have different ways of escaping from the immune system. Therefore, the immune system loses the necessary effectiveness against the parasite.

In the present study, somatic and secretory antigens of adult *Fasciola spp.* were extracted and, after evaluating and comparing their protein profiles, injected into white rabbits, then the blood serum of immunized rabbits was tested by Western blotting for immunogenicity. According to the results of Western blotting, 11 bands of somatic antigens with a molecular weight of 149, 122, 99, 85, 75, 65, 50, 46, 40, 37, 30 kDa and 12 protein bands of excretory/secretory antigens with a molecular weight of 82, 100, 75, 70, 58, 55, 47, 40, 38, 37, 30, 25 KDa of adult *Fasciola spp.* were observed immunogenic which appear to have a protective effect or may be used to prepare a suitable diagnostic kit. Certainly, research on these antigens can be an effective way to achieve this goal.

Authors' Contribution

Study concept and design: Am. D., A. D, N. H. R., and R. M.

Acquisition of data: Am. D.

Analysis and interpretation of data: Am. D., A. D., and R. M.

Drafting of the manuscript: A. D. and R. M.

Critical revision of the manuscript for important intellectual content: A. D.

Statistical analysis: A. D.

Administrative, technical, and material support: A. D., N. H. R., and R. M.

Ethics

This study was confirmed by the Ethical Committee of Islamic Azad University University.

Conflict of Interest

The authors declare that they have no conflict of interest.

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