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Determination of the electrophoretic pattern of somatic and excretory-secretory proteins of *Ligula intestinalis* parasite in spirlin (*Alburnoides bipunctatus*)

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Abstract. *Ligula intestinalis* parasite is a cestodes that causes remarkable damages to fish. It is also of prime importance in economic and hygienic aspects. SDS-PAGE and western blotting are the methods that can be used to determine the electerophoretic pattern of somatic and excretory-secretory proteins of parasites. In this study, after obtaining the plerocercoidal stage of this parasite from the spirlin (*Alburnoides bipunctatus*), its somatic proteins were prepared using ultrasonicae, and excretory-secretory proteins were prepared using the PBS solution. After protein assay, which included using the Bradford method and then SDS-PAGE on these two antigenic solutions, 5 protein bands of 26, 33, 38, 58, 70kDa in somatic antigens, and 7 bands of 25, 28, 33, 43, 49, 60, 70kDa in excretory-secretory antigens were observed. After western blotting on both antigens and adding the primary antibody (the sera of infected fish) and then the secondary antibody (Rabbit Anti-fish Polyclonal Antibody Conjugated from Abnova Corporation) no band was seen in excretory-secretory antigen. And only in the 55kDa band of somatic antigen, a positive response, in comparison of fish positive serum was observed.

INTRODUCTION

Ligula intestinalis is a member of the Ligulidae family of cestodes. The mature worm lives in the intestine of fish-eating birds, and has two intermediate hosts in its life cycle. The first host is copepods which live in fresh and or salty waters in which the first larval stage of the parasite is formed. The second intermediate host for this parasite are freshwater fishes especially Cyprinidae (Dubinina, 1980). The parasite is seen as plerocercoid larvae which is in the abdominal cavity area and causes ligulosis disease in the fish (Hoole *et al.*, 2010). Sterility of infected fish is due to specific interactions of the parasite with the fish (Arme, 1997; Carter *et al.*, 2005; Trubiroha *et al.*, 2009). In this study, excretory- secretory proteins (proteins that are expelled from the parasite body after metabolism, meaning that the host also plays a role in this form of protein, and *L. intestinalis* parasite also gets food from the host body) and somatic protein (proteins which are in the body of the parasite without any interference from the host) of plerocercoidal stage of *L. intestinalis* parasite were obtained from spirlin (*Alburnoides bipunctatus*) which were assessed for determining molecular weight and the dominant immunogenic antigen. The aim of this study was primarily to determine the electrophoretic pattern of the plerocercoidal stage of *L. intestinalis* and then to determine the most important antigenic fraction of the parasite against the positive serum of the fish infected with *L. intestinalis*.

MATERIALS AND METHODS

Preparation of the samples

In this study, 122 fish (mean weight 18±2 gr, mean length 10±2 cm) were taken and 66 fish were infected by *L. intestinalis* parasite. In each infected fish, 2 parasites (mean weight 3±1 gr) were separated. To determine the electrophoretic pattern of excretory-secretory and somatic proteins of *L. intestinalis*, first, the parasite was separated from the abdominal cavity of infected spirlin (*A. bipunctatus*) and blood samples were taken from the fish by caudal puncture. Then, blood samples were centrifuged at 2000(g) for five minutes, and then their serum was separated and evaluated by ELISA procedure. Afterward, the sera with high titration (proper for fish positive serum) were selected and stored in -70 degrees centigrade (this is the primary antibody for western blots). After separating the parasites from the abdominal cavity area of the fish, and in order to prepare excretory-secretory proteins, the parasites were kept in a 5^{cc} PBS solution for 24 h in an incubation condition of room temperature near to fish body's temperature. After bringing out the proteins from the PBS solution, and by using dialysis sac, proteins were concentrated as far as possible. Then, using ultrasonicate, the parasites were sonicated at 110 V, 170 W ultrasonic disintegrator (Hielscher, Germany), for 3×15seconds on ice to obtain somatic antigens. The prepared solution was then left on ice for one hour. Finally, and using a refrigerator centrifuge, the prepared excretory-secretory and somatic proteins were centrifuged for 30 mins at 5000(g) at 4°C and the supernatant was collected. After protein assay using the Bradford method (Bradford, 1976) the electrophoretic pattern was determined by SDS-PAGE and then western blotting.

SDS-PAGE and western blotting

The prepared antigens were run on SDS-polyacrylamide gels, composed of 5% resolving gel and 10% stacking gel, under reducing conditions using the discontinuous buffer system (Laemmli, 1970). For size estimation in SDS-PAGE, a pre-stained protein marker at a range of 10.5-175kDa molecular weight (pr0602) was used. Proteins were transferred in a Bio-Rad Trans-Blot Cell for 12 h in a constant current of 30 V. For size estimation in western blotting, a pre-stained protein marker at the range 10-160 kDa molecular weight (SM-0671) was used. Blocking in 3% dry skimmed milk in PBS containing 0.1% Tween 20 was carried out 1 h at room temperature. Strips were washed three times with a PBS containing 1% Tween 20 for 10 mins and then incubated with the first antibody diluted in PBS 0.1% Tween 20 for 1 h at room temperature. The strips were washed again for 3×10 min and incubated with the secondary antibody [Rabbit Anti-fish Polyclonal Antibody Conjugated from Abnova Corporation that this antibody is conjugated to DAB (diaminobenzidine tetra hydro-chloride) and against fish IgM] for 1 h at room temperature. After incubation, the strips were washed as before in PBS 0.1% Tween 20. 3, 3´- diaminobenzidine tetra hydrochloride (sigma) substrate was added to 25 ml of PBS and then 10 µl of H2O2 30% was added for exactly 2-5 min at room temperature. Finally, the reaction was stopped by the addition of distilled water to strips (Towbin *et al*., 1976).

RESULTS

Protein assay

After the protein assay, which used the Bradford method, the somatic antigens protein measure was 90 micrograms per milliliter, and the excretory-secretory proteins measure was 35 micrograms per milliliter in volume unit.

SDS-PAGEAfter performing SDS-PAGE on both somatic and excretory-secretory antigens, 5 bands (26, 33, 38, 58, 70kDa) were seen in somatic antigen in which the 33 and 38kDa bands were more prominent. Seven bands (25, 28, 33, 43, 49, 60, 70kDa) were also noticed in excretory-secretory proteins, in which the 25 and 33kDa bands were more prominent (Figure 1). These results show that somatic proteins had less band separation due to their higher protein concentration in comparison to excretory-secretory antigens in SDS-PAGE.

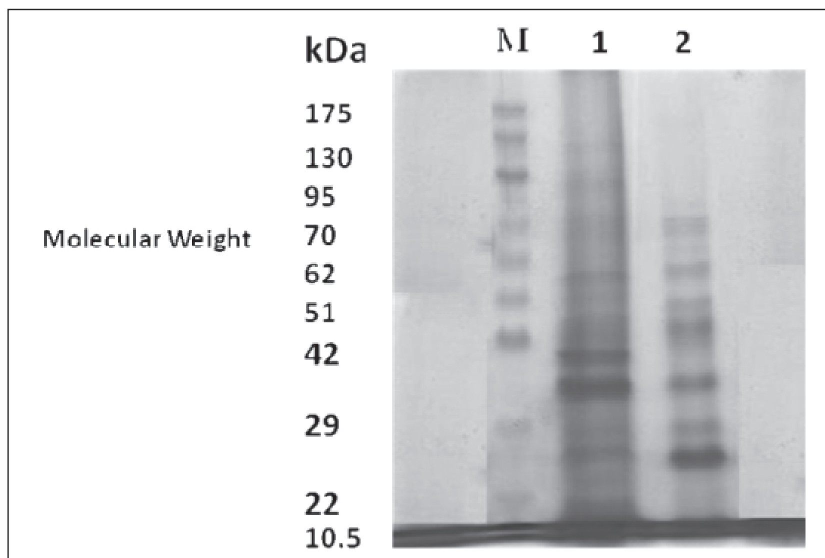

Figure 1. Results of the SDS-PAGE of somatic and excretory-secretory proteins. M=Marker, 1= Somatic Antigen, 2= Excretory-secretory Antigen

Western blotting

After transferring proteins from the gel to nitrocellulose paper, using the blotting technique, no bands were detected in excretory-secretory antigens against infected fish serum, but in the somatic antigen's column, a band of 55kDa was detected, which shows a high antigenic effect of this protein in somatic antigen of parasite against fish serum (Figure 2).

DISCUSSION

In the study conducted by Williams & Hoole (1995), which is the only study conducted on *L. intestinalis* using SDS-PAGE and western blotting, only normal serum [normal roach serum (NRS)] and serum contaminated with *L. intestinalis* immune roach serum (IRS) in roach (*Rutilus rutilus*) was assessed. However, bands of 65, 90 and 100kDa were shown in the SDS-PAGE of both sera (NRS and IRS) in which the IRS bands were sharper than NRS bands. After western blotting and the adding of conjugated antibody, the 65 and 90kDa bands were seen in both NRS and IRS. But in the present study, in the SDS-PAGE of somatic proteins, the 26, 33, 38, 58 and 70kDa bands, and in excretory-secretory proteins, the 25, 28, 33, 43, 49, 60 and 70kDa bands were observed. After western blotting, the only band which was observed in somatic antigen was the 55kDa, and no band in excretory-secretory antigen reacted to infected fish serum.

Lightowlers & Rickard (1988) studied the role of excretory-secretory products of intestinal worms on the host's immune system. They declared that it is possible that excretory-secretory products have an effect on the host's immune system. According to the results of the present study, it has been shown that excretory-secretory products of *L. intestinalis* do not have a significant effect on activating antibody response in spirlin (A. *bipunctatus*), but one of the fractions of somatic antigen (55kDa band) has the capability of activating antibody response in the mentioned fish.

Hoole & Arme (1986) studied the role of serum in adhesion of leukocytes which were exposed to a plerocercoid form of *L. intestinalis*, in which after adding roach antibody, adhesion was observed in the leukocytes which were exposed to the parasite. So in this study, roach has shown immune response to the parasite. In the present study, 55kDa proteins of somatic antigen are capable of activating antibody response in spirlin (*A. bipunctatus*) as well.

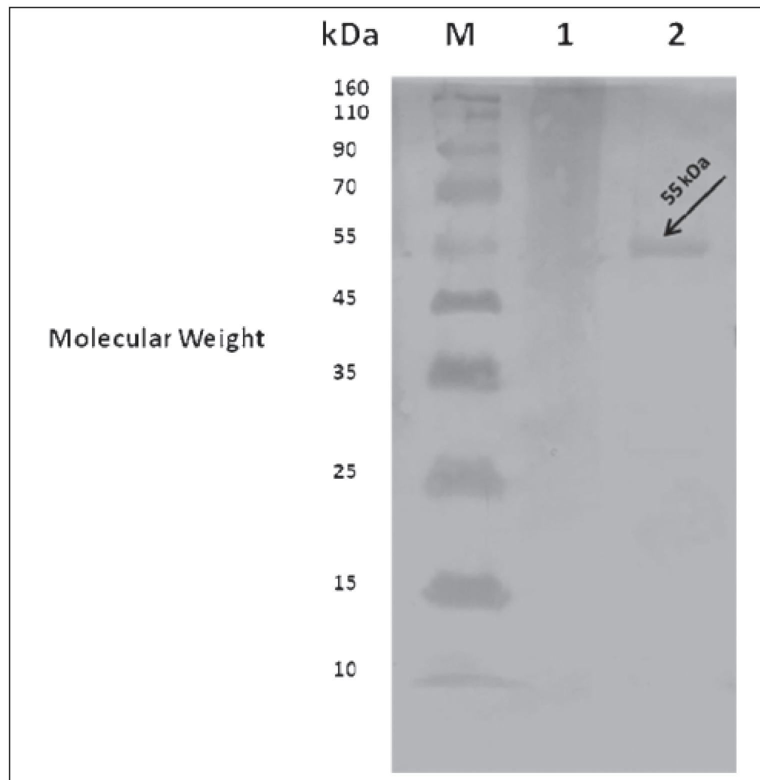

Figure 2. Results of the western blotting of somatic and excretory-secretory proteins. M=Marker, 1= Excretory-secretory Antigen, 2= Somatic Antigen

Molnar & Berczi (1965), using a double diffusion test, showed that the serum of *Abramis brama* infected with *L. intestinalis* contains a precipitin against *L. intestinalis*. Williams & Hoole (1992) also, using the mentioned technique, showed that roach makes a specific antibody against *L. intestinalis*. Considering the higher sensitivity and specificity of SDS-PAGE and western blotting in comparison to double diffusion, these two techniques are capable of showing important antigenic fractions against infected fish serum, which as it was shown in the results, the 55kDa protein fraction of somatic antigen showed the best response to infected fish serum.

Arme & Owen (1968, 1970) stated that in most cyprinids, Ligula's plerocercoid causes a more severe immune response, which in *R. rutilus* involves macrophages, neutrophils, monocytes, granulocytes and lymphocytes (Hoole & Arme, 1982,1983a,b). According to our results of western blotting of *L. intestinalis* somatic antigens, the 55kDa proteins were also able to activate an antibody response in spirlin (*A. bipunctatus*) which means they can be used to immunize this fishes.

In conclusion, considering the fact that different lab diagnostic tests have different specificities and sensitivities in detecting of contamination, we decided to use SDS-PAGE and western blotting to determine the electrophoretic pattern of *L. intestinalis* plerocercoidal stage in spirlin (*A. bipunctatus*). As it was shown in the results of this research, the 55kDa fraction of somatic antigen was the only fraction capable of activating the fish's immune system and showed a positive response against positive serum of the fish. Meaning that the somatic antigen, was the most important protein of *L. intestinal is* body's and could evoke an immune response in the host *in vivo*. So using these results, a more proper treatment method can be applied against this parasite.

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