

Original Article

Determination of Immunogenic Relevant Antigens in the Excretory-Secretory (ES) Products and the Lysates of *Ascaridia galli* Larvae

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Abstract

Background: *Ascaridia galli*, the largest nematode of small intestine of birds, especially the native poultry, may give rise to serious illness, pathological defects and economical losses even in modern poultry production systems. Although various measures have been undertaken to vaccinate poultry against *A.galli*, no satisfactory results were obtained so far. However, there is no report on the efficacy of excretory-secretory (ES) proteins of *A.galli* larvae in immunization of poultry. Thus, the aim of the present research project was based on the use of the ES products of the larvae, in order to find the protective antigens.

Methods: Five hundred native poultry were autopsied and adult *A.galli* was removed from their intestines. The eggs were harvested from the uterus of female worms and cultured at 25 °C in water containing 0.1 N sulphuric acid for almost a fortnight. The larvae were then freed mechanically and kept in Earl's salt solution for a few days. The supernatant solution of alive larvae containing the ES products of the larvae, as well as the sonicated alive and dead larvae, was analyzed by SDS-PAGE.

Result: Many protein fractions of 15 kDa up to 200 kDa were demonstrated in lysate of these larvae. Using the serum of a hen, infected with a high numbers of *A.galli*, an immunogenic antigen was identified between 55 kDa to 72 kDa by Western blotting procedure.

Conclusion: Finding the protein band between 55 and 72 kDa can be promising for preparation of vaccine, though more investigations are needed to prove the protective ability of this antigen.

Keywords: *Ascaridia galli*, Excretory-Secretory antigens, SDS-PAGE, Western-blot

Introduction

A *scaridia galli* is one of the largest nematode of the small intestine of birds. It may cause reduction in growth rate, weight loss, sometimes serious illness, pathological lesions and especially economical losses in native birds such as hens, turkeys, geese and some other birds (1). Also, damage to the intestinal mucosa leading to blood loss and secondary infection and occasionally the obstruction of small intestine of poultry due to high worm burden may occur (2).

Meanwhile *A.galli* is prevalent in Iran and its prevalence varies from ten to forty four percent among the native and industrialized poultry in different areas of this country (3). Recently, Permin (personal communication, 2008) estimated the economic loss due to *A.galli* in free range and deep litter chickens to be 20% approximately. The economic impact of *A.galli* and the consequences of antiparasitic treatments from chemical residues in food products and the environment as well as the occurrence of drug resistance has led to an increasing interest to

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develop vaccine for controlling the infection in poultry. Thus, Brewer and Edgar used known quantities of whole worm as well as the embryonated egg protein which only engendered partial immunity, light increase on weight gain and protected birds against worm numbers (4). Also, many research works were carried out to protect hens against this nematode but no satisfactory results were obtained, i.e., use of irradiated eggs to develop immunity (5), the role of CMI which was highlighted in protection against *A.galli* infection with irradiated infective eggs (6, 7), repeated challenge of poultry with infective eggs (8), placing chickens on different levels of nutrition (9) and report of partially resistant age and breed of poultry to *A.galli* infection (10-12). It was shown that any resistance to this nematode infection depends on the breed, age and nutritional status of the bird (11, 12). Meanwhile the impact of trace elements and the use of somatic antigens, prepared from adult worm, was not successful in vaccination (13-15). Abdel-Rahman and Khalil have tried to analyze the excretory-secretory (ES) as well as somatic antigenic pattern of the adult *A. galli* and could find in the lysates and supernatant of cultured adult worms some protein bands (16). However, there is no report of the lysates derived from the larvae or larval ES products of *A. galli*. Due to the importance of larvae which hatches from the embryonated egg in the susceptible bird intestine and its penetration into the intestinal mucosa, as a first step for establishment of the parasite in the host, we tried to clarify the protein profile of the ES products, as well as the lysate of alive and dead larvae by SDS-PAGE and Western blotting methods. We hope that the immunogenic antigens from ES may help us to develop adequate protection in poultry.

Materials and Methods

Preparation of *A. galli* eggs

A. galli were collected from the intestine of 500 hens of different areas of East Azerbaijan in

Iran. The adult worms were collected and characterized using the identification key of Eslami (3). The worms were washed in physiologic saline (0.9% NaCl solution) and their uteri were then separated and the eggs were extracted by pressure through the forceps.

Culturing the eggs and preparing antigens:

The eggs were cultured in 0.1 N sulphuric acid in tap water at different temperatures, i.e., room temperature, 18 °C and 25 °C (17), until the embryos were developed inside the eggs and were visible under the light microscopy, within 10-25 days. The eggs were then washed three to four times with PBS, followed by Earl's balanced salt solution (18, 19).

In order to make free larvae from the embryonated eggs, we performed a mechanical procedure using a rounded tip glassy rod. The larvae, either alive or dead were then collected using pipette and transferred to the Earl's solution. The alive larvae were kept at 25 °C, as long as they were moving within 48 to 72 hours. Then tubes containing these larvae were centrifuged at 1500 rpm for 5 minutes and the supernatant fluids were collected and kept at -20 °C until used. Meanwhile, lysate of the alive larvae and all dead larvae, in addition to embryonated eggs, were prepared by sonication. The larvae and eggs were sonicated by 0.5 cycles and 80% amplitude for 1.5 minutes with five times repeating in two minutes interval using sonicator (Dr. Hielscher, Germany). The lysate were then stored at -20 °C.

Collection of sera from infected hens with *A. galli*

The sera of 20 hens of 2.5–3 kg body weight and more than one-year age, which were infected with *A. galli*, were collected, according to the routine procedures of hematology. The sera were kept at -20 °C.

Preparation of Rabbit anti hen-Immunoglobulins

One native hen of 2.6 kg and 17 months old was bled. Immunoglobulin from serum was prepared according to the preparation of chicken γ -globulin (20). The serum from the co-agglutinated blood was separated by centrifugation. Then the bird was sacrificed and its

intestine was opened and checked for the absence of *A. galli* worm.

The hen's serum was first diluted to one-half by physiologic serum. Then the saturated ammonium sulphate was added to achieve the end concentration of 45%. The solution was incubated at room temperature for 30 min. under shaking condition and then the solution was centrifuged by 1000 x g, at 4 °C for 15 min. 45% ammonium sulphate was added to the pellet and mixed thoroughly and then centrifuged by 1000 x g, at 4 °C for 15 min. The supernatant was transferred to the clean tube. Saturated ammonium sulphate was then added to the supernatant until the concentration of ammonium sulphate was 40%. After that the solution was centrifuged by 1000 x g, at 4 °C for 15 min. The pellet was then washed with 40% ammonium sulphate and the resulting pellet was resolved in PBS. 0.5 ml of purified immunoglobulins mixed with 0.5 ml Complete Freund's adjuvant were injected to rabbit subcutaneously. Another 0.5 ml purified immunoglobulins mixed with 0.5 ml Incomplete Freund's Adjuvant were injected to the same rabbit subcutaneously, after 3 weeks. In order to be assured the high level of antibodies in the rabbit serum after 3 weeks, the Ochterlony plate was used. The specific rabbit serum was used in Western blotting procedure.

SDS-poly acrylamide gel-electrophoresis (SDS-PAGE)

The SDS-PAGE method was performed according to the procedure described by Laemmli (21). For the separation of antigens, 5% concentrating gel followed by 12% separating gel was used. The visualization of the proteins on the gel was

performed using Coomassie blue staining method.

Western blotting method

The proteins in the gel were transferred to the nitrocellulose membrane by constant 15 volt using buffer system recommended by semi-dry blotter (BioRad). The immunogenic antigens were then detected using serum from infected hen with *A. galli* in peroxidase system.

Results

Culture of the *A. galli* eggs was suitably performed in tap water containing 0.1 N sulphoric acid at constant 25 °C of incubator. The maximum rate of the egg embryonation was 70% after 10-11 days. Ascertaining this time, which was resulted after several assays, had two advantages, firstly we could arrange next step of the procedure in due time, and secondly we could collect the most active and viable larvae by mechanical stimuli.

Analysis of lysate of 20 µl of 200 alive larvae (400 µg/ml) as well as many dead larvae and the embryonated eggs, by the SDS-PAGE, revealed 15, 20, 25, 30, 40, 50, 60, 70, 85, 100, 120, 150 and 200 kDa bands (Fig. 1). The protein bands of 55 to 72 kDa were observed in the analysis of somatic antigens of the alive and dead larvae with the positive serum of a hen infected with *A. galli* adult worms (Fig. 2). The SDS-PAGE analysis demonstrated no protein band in the ES products of the alive larvae due to the low concentration of protein in the solution. However, Western blot analysis showed that at least one prominent immunogenic band between 55 to 72 kDa was detectable, using serum from infected hens with *A. galli* adult worms (Fig. 3).

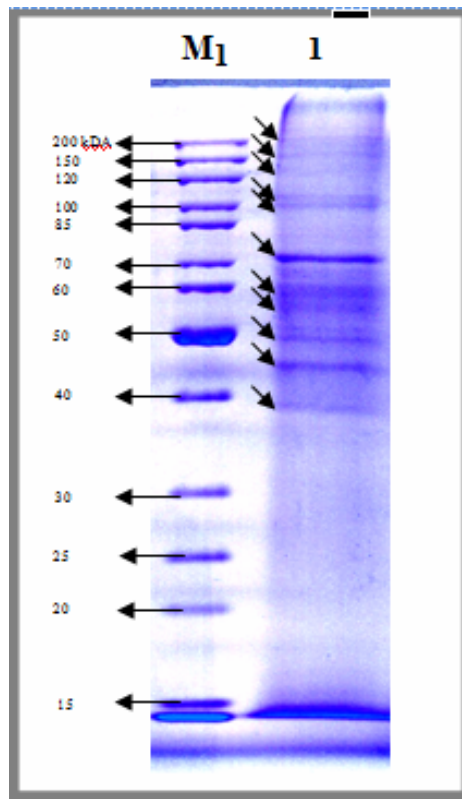


Fig 1: Band 1. The somatic antigens of 200 viable larvae as well as many dead and embryonated eggs of *Ascaridia galli*. M1 : the ordinary marker, Fermenta No. 0661

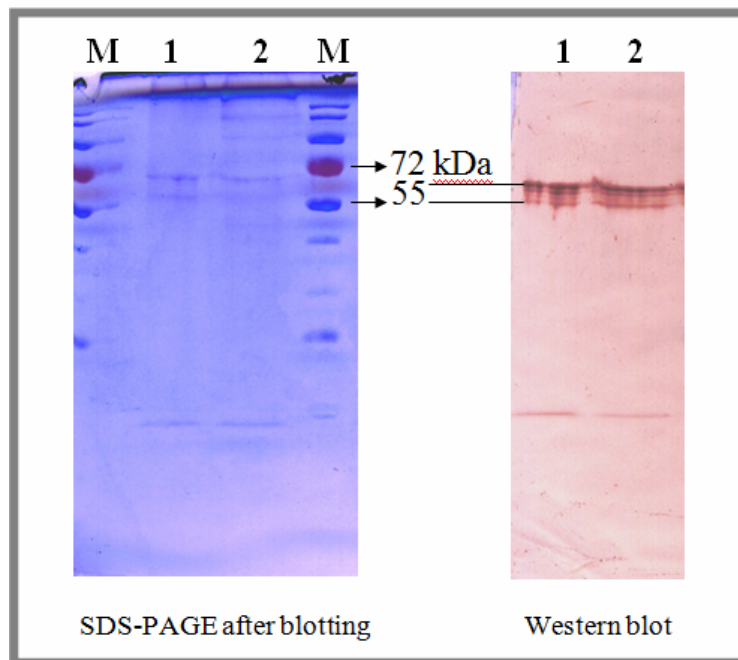


Fig 2: Band 1 - The somatic antigens of viable larvae of *Ascaridia galli*. Band 2 – The somatic antigens of the dead larvae of *A. galli*. The somatic antigens of alive and dead larvae were analysed with the positive serum of a hen infected with *A. galli* adult worms. M – prestained marker.

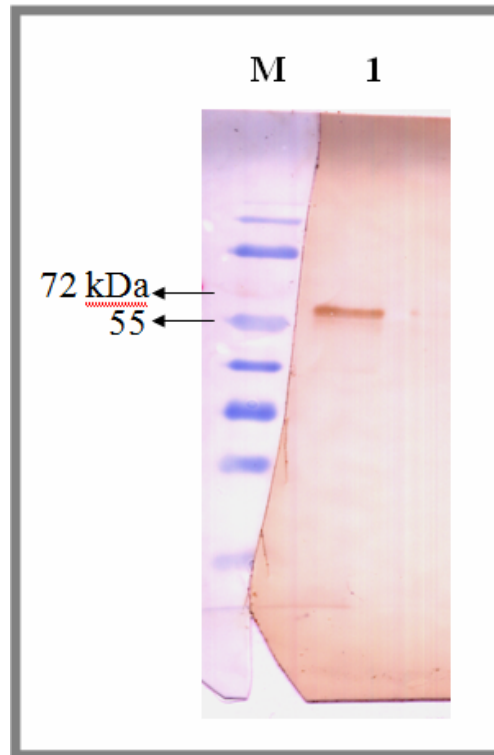


Fig 3: Band 1 – The secretory-excretory (ES) products of the larvae analysed with the serum of a hen infected with *A. galli* adult worms. M – Prestained marker.

Discussion

We could free larvae using the digestive enzymes (pepsin and trypsin), the bile of hens, the CO₂ gas pressure, and the appropriate temperature in a suitable redox condition(22-24), but we preferred not to use the artificial hatching process in vitro, for avoiding the intervention of host proteins into the ES products of the freed larvae and their related protein profile. Thus, we made free the alive larvae by mechanical procedures, and then the supernatant solution of the alive larvae were collected and analyzed by SDS-PAGE. The specific proteins were identified by Western blotting method as 55 KDa up to 72 kDa bands, using the positive sera of hens harbouring high worm burden of adult *A. galli* in their intestines. It seems that 55 kDa up to 72 kDa proteins may be immunogenic/protective antigens of this worm, though more studies are needed to prove this. Meanwhile, the alive, dead larvae and the embryo-

nated eggs were sonicated and their lysate was analyzed by SDS-PAGE. The protein bands were identified as 40, 40–50, 50-60, 60-70, 70-85, 100, 120-150, 150-200 and 200 kDa (Fig. 1). Comparison of the present findings with similar data of other workers was not satisfactory for, surprisingly, there was no report related to the ES products of *A.galli* larvae (Permin, personal communication). Nevertheless, the report of Abdel-Rahman and Khalil (16) who analyzed the lysate of adult male and female *A.galli* by SDS-PAGE method, with the molecular weight of 41, 76, 103, 110, 157, and 207 kDa, corresponds more or less to the findings of the present study. When the infective egg is swallowed by the bird, it hatches and the larva will be freed in the intestinal lumen. Then the larva has to interact with the intestinal mucosa of the host and penetrates into it (10, 13, 17). In immunized host, the larva fails to invade the intestinal mucosa (2). Attempts were paid to find out the appropriate antigens competent enough to provoke the host's

immune system. Thus, the protein profiles of adult male and female of *A.galli* and the ES products of mature worms were analyzed and the host specific fatty acid-binding proteins (FABPs), a member of the nematode polyprotein allergen family were reported (25). Lipid binding proteins exhibit important functions in lipid transport, cellular signaling, gene transcription and cytoprotection in nematodes, therefore, they are called nematode polyprotein allergens/antigens (NPAs) and fatty acid and retinoid-binding proteins. Jordanova *et al.* (14) purified a novel 55 kDa protein called as Ag-1 bp 55 from *A.galli*. These workers reported that Ag-1 bp 55 (55 kDa) is highly abundant protein mainly distributed in the inner hypodermis and extracellularly in the pseudocoelomic fluid. Parasitic nematodes have to adapt to different conditions during their life cycle. The free-living stages utilize endogenous carbohydrate reserves and are to metabolize lipids aerobically for energy generation, whereas the adult parasitic stages are solely dependant on carbohydrate metabolism as an energy source. However, due to constant turnover of structural lipids, the adult nematodes synthesize triacylglycerols and furthermore certain lipid derivatives, i.e., peroxides, steroids and prostaglandins (25). We presume that the protein band, specified in the supernatant fluid of alive larvae in our study, might be partially identical to 55 kDa (Ag-1bp 55) reported by Jordanova *et al.* (14). At present, we have no information about the antigenicity and/or protectivity of this protein that is a subject of more investigations to be carried out in future.

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