A Preliminary Study on the Antigenic Proteins of

*Linguatula serrata* Nymphal Stage in Sheep with Visceral Linguatulosis

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Abstract

Scant information is available on the immunological aspect of *Linguatula serrata* causing linguatulosis in humans and animals. The purpose of this study was to analyze the content of crude somatic extracts (S) and excretory-secretory products (ESP) of *L. serrata* nymphs to detect the immune response of sheep and immunogenic proteins of the parasite. After collecting the nymphs, somatic extracts (S) were prepared by sonication. Excretory secretory products (ESP) were prepared by incubating nymphs in RPMI medium at 37°C with 5% CO2. Somatic and excretory-secretory proteins were isolated with SDS-PAGE. Immunogenic properties of the resulting proteins were determined using immunoblotting and positive sera from sheep infected with visceral linguatulosis. The total content of somatic extracts (S) and excretory-secretory products (ESP) of *L. serrata* nymphs analyzed by SDS-PAGE (12% gel) revealed two protein patterns with more than 18 and 9 strong bands, respectively. Immunoblots using sera samples of sheep infected with the parasite, S and ES materials demonstrated 12 and 3 antigenic proteins with molecular weights mostly in the range of 24 to 100 kDa and an antigen more than 180 kDa. Three common immunodominant antigenic proteins with a molecular weight of 38, 57, and an antigen of more than 180 kDa, showed in the
somatic extracts (S) and excretory-secretory products (ESP) of *L. serrata* nymphs in sheep with visceral linguatulosis. These antigens can be mentioned as a prime candidate for future serodiagnosis and immunoprotective studies of the parasite.

**Keywords:** *Linguatula serrata*, Antigen, Immunoblotting, Sheep.

**INTRODUCTION**

*Linguatula serrata* (Frohlich, 1789) a parasitic arthropod from the phylum called Pentastomida (Shipley, 1905), the family Linguatulidae (Haldeman, 1851) is a zoonotic parasite with an indirect life cycle. Adults live in the nose of dogs (and rarely of men), the length of male and female is about 2 and 12 cm, respectively. Embryonated eggs are released via nasal discharge and/or feces. If intermediate hosts (including cattle, sheep, goats, and buffalo) swallow eggs, the larva hatches and migrates via blood vessels to viscera (including mesenteric and hepatic lymph nodes, liver, lung, etc. (1, 2). Human linguatuliasis is reported from certain parts of the world, especially in countries of the Middle East, Southeast Asia, Africa, and America (3, 4, 5-8). The prevalence of linguatulosis in human populations is also unknown simply because infections can only be diagnosed through rare ocular involvement (9,10) and incidentally discovered at laparotomy or autopsy (5, 6, 11). There are several notable reports of linguatulosis in humans from different provinces of Iran (12-15). Respiratory ducts especially nasal cavities are the habitat of the adult parasite in canine and feline species (2). The prevalence of linguatulosis in dogs varies considerably throughout the world and is reported 76.5% in stray dogs of Iran (16), 38% in India (8), and 53% in Turkey (17). The infected dogs usually lack specific clinical signs, therefore the diagnosis of linguatulosis in these animals was detected mainly at the surgery or
autopsy by isolation of the parasite from some organs such as liver, lungs, and lymph nodes (18).

Natural infection of *L. serrata* in all domestic ruminants and some carnivorous animals has been reported from different geographical parts of Iran (19, 20, 16, 21-26). The importance of Ab/ or Ag detection in serological methods is very clear for the diagnosis of infections.

Moreover, serological assays could also allow the processing of many samples in a short time, which would be useful in health management. Since adequate information on excretory-secretory (ESP) and somatic (S) antigens of *L. serrata* is not yet available, therefore, the current study was designed to characterize *L. serrata* antigens, which may be used as a basis for future researches on the specific immunological diagnosis of visceral linguatulosis in sheep and other animals.

**MATERIALS AND METHODS**

*Sera and L. serrata nymph collection*

Positive control sera were prepared from the sheep that were selected to slaughter. Blood sampling was performed before slaughtering the animals in the slaughterhouse. The mesenteric lymph nodes of slaughtered sheep were separated from the body and examined directly or by stereomicroscope for nymphs of *L. serrata*. The serum of any animal in which the parasite (nymph) was observed in its lymph nodes, considered as positive serum. Collected nymphs were washed several times with tap water, then used for somatic extract or excretory-secretory products. Negative sera were prepared from indoor lambs.
Preparation of S and ES proteins

Collected nymphs of the parasite were washed separately 3 times with 0.85% saline (sterile) and phosphate-buffered saline (PBS, pH=7.2, supplemented with 100U/ml of penicillin G, potassium and 100 mg/ml of streptomycin. The viability of the nymphs was checked under a stereomicroscope. For the preparation of crude somatic extract (S), a total of 250 nymphs were triturated by scalpel, homogenized by ultrasonic homogenizer (Bandelin, Berlin, Germany) in 10 mL mixed of PBS and RPMI (with the same proportion), and then centrifuged at 2000 g for 10 min at 4°C (to eliminate cell debris). The extract was filtered through 0.22 mm filters (Biofil Syringe Filter).

The excretory-secretory product (ESP) were obtained from the culture in vitro of the parasite nymphs. A total of 250 nymphs were placed in a 75 cm² cell culture flask (Greiner Bio-One, Solingen, Germany), containing 10 ml RPMI-1640 (Bahar Afshan, Iran) with 100U/ml of penicillin G potassium and 100 mg/ml of streptomycin. The flask was incubated in darkness for 24 h in a 5% Co2 atmosphere at 37°C. Supernatants were collected, centrifuged at 2000 g for 10 min at 4°C, and filtered through 0.22 mm filters. To increase the protein concentration, filtrates were concentrated up to 3 times (3X) under nitrogen flow.

Protein concentrations of the S and ESP filtrates were determined by the Bradford method and stored them at -20°C until use.

SDS-PAGE and Immunoblotting

SDS-PAGE of somatic and excretory-secretory proteins was performed in different percentage polyacrylamide gels (4% stacking, 12% resolving gel) using a discontinuous system as described by Laemmli (27). Briefly, 60 µl of each sample were mixed with
30 µl of a non–reducing sample buffer [SDS (10%), Tris-HCl (1M; pH 6.8), glycerol (2%)], boiled for 5 minutes, centrifuged for 1 min and supernatants used for loading. For molecular weight estimation, a pre-stained protein marker broad range (25-180 kDa) from BioRad was also subjected to gel electrophoresis, which was carried out using a Mini-Protean III Cell (Bio-Rad) at 100 V constant voltage for 5 hr. The gel was stained for protein visualization with Coomassie blue R-250 (0.1% w/v).

For immunoblotting, proteins of S and ES products were electrophoresed on 12% SDS-polyacrylamide gels. Proteins from SDS gels were electrotransferred onto nitrocellulose membranes (NC) using a Trans-Blot Cell (Bio-Rad) and transfer buffer (Tris 25 mM, glycine 25 mM, pH 9.0) for 3 hr. at 60 V. Afterwards, the transferred proteins were blocked with blocking solution (5% skim milk in PBS) at room temperature for 1 h.

NC strips were washed (2-5 min) in the washing buffer (PBS-T: PBS, 0.05% Tween20) and incubated for 1–2 h with specific anti-L. serrata sheep sera that confirmed by ELISA test (n=36). Control tests were carried out with sera from non-infected lambs (n=4). Four dilutions of sera (1:10, 1:20, 1:40, and 1:80) in diluting buffer (PBS-T, 5% skim milk) were examined 4 times and chosen better dilution for continuing the tests.

The strips were washed again (2-5 min) in PBS and developed with anti-sheep IgG peroxidase-conjugated (Sigma- Aldrich, USA) in dilution 1:1000 for 1 hr at 37ºC and in a mixture of H2O2-Chlornaphtol for 15 minutes.

RESULTS

Protein concentrations of S and ESP were obtained 90 and 30µg /ml, respectively. The total somatic extracts analyzed by SDS-PAGE (12% gel) revealed more than 18 protein
bands with molecular weights of 25 to more than 180 kDa (some predominant bands including, 25, 32, 36, 45, 48, 57, 67, 70, 75, 84 and 100 kDa) (Fig 1).

The Coomassie blue-stained SDS-PAGE of ESP of L. serrata nymphs revealed more than 9 polypeptides, from 24 to more than 100 kDa. Polypeptides with molecular weights of 28, 35, 48, 57, and 74 kDa were the most predominant bands of them (Fig 1).

Analysis of electrophoretic patterns in immunoblotting of somatic extracts (S) and ESP of L. serrata nymphs with the 1:10 dilution of positive serum samples (from naturally infected to L. serrata) and 1:1000 dilution of anti-sheep IgG conjugate, positive reactions (as visible bands) were obtained. The results revealed 12 antigenic polypeptides (bands) in somatic extracts (S) of L. serrata nymphs with apparent molecular weights of 28, 38, 49, 57, 63, 67, 91, and one band higher than 180 kDa (Fig 2).

The results revealed three main proteins (bands) in ESP of L. serrata nymphs with apparent molecular weights of 38, 57 and one specific polypeptide band (like to S) with a molecular weight of higher than 180 kDa (Fig 2).

**DISCUSSION**

The importance of linguatulosis in veterinary and medicine is considered in the world including Iran. The eggs of L. serrata, which contain larvae are ingested by different vertebrate intermediate hosts including ruminants and man (1,2).

Clearly, there is a need for a reliable serodiagnostic test to establish the prevalence of pentastomiasis in men, dogs, and livestock. Antibodies usually reflect the contact of the host with the parasite and could also reflect the infective status of an animal. Serological testing of linguatulosis can be the most practical method for monitoring the exposure status of farms to implement control measures.
Scant information is available on the immunological aspect of *L. serrata*. Our study showed the presence of more than 18 protein bands (some of them with molecular weights of 25, 32, 36, 38, 45, 48, 55, 57, 67, 70, 75, 84, 91, 100, 110, 135) in somatic extracts of *L. serrata* nymphs SDS-PAGE profile. The study of Hajipour et al. 2016, is the only available study on the profile of somatic proteins of *L. serrata* nymphs (28). In this study, they found the presence of 6 similar protein bands (14.4, 32, 36, 48, 75, and 100 kDa) in somatic proteins of the nymphal stage of *L. serrata* collected from goats and cattle (with the additional band, 120 kDa in cattle). In comparison to our study, the controversy results in protein profiles of *L. serrata* nymphs obtained by Hajipour et al. 2016 can be due to the differences in methods and type of hosts (28).

In the present study, attempts were made for the determination of diagnostic antigens of *L. serrata*, which could be used for the specific immunological diagnosis of linguatulosis. Diagnosis of infection by demonstrating specific antibodies in an infected animal relies on the use of good quality antigen. Furthermore, a detectable and persistent antibody response in all infected animals to an antigen is the ideal choice for serological diagnosis of infectious diseases (29). Based on the results of the present study, immunoblot analysis of S and ES with sera of ovine infected with *L. serrata* showed reactivity with twelve and three main proteins, respectively. Among the bands, three common immunodominant antigenic proteins with a molecular weight of 38, 57, and an antigen of more than 180 kDa were recognized in the somatic extracts (S) and ES products of *L. serrata* nymphs by the sera of sheep with visceral linguatulosis. Alborzi et al, 2015 evaluated an ELISA test for serodiagnosis of the parasite infection in sheep by using somatic extracts (S) and excretory-secretory products (ESP) of *L. serrata*. Although in their study, the results of the sensitivity, specificity of the ELISA
were almost the same for S and ES, ES antigens were better than S antigens for detecting the infection in sheep (30). The findings of both studies reflect the similarity of antigens in the somatic extracts (S) and ES products of the parasite and also confirm the common immunodominant antigenic proteins determined in our study.

**Conclusion**

Conclusively, in our findings, three common immunodominant antigenic proteins with a molecular weight of 38, 57, and an antigen of more than 180 kDa, showed in the somatic extracts (S) and excretory-secretory products (ESP) of *L. serrata* nymphs in sheep with visceral linguatulosis. These antigens can be mentioned as a prime candidate for future serodiagnosis and immunoprotective studies of the parasite.

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**Conflict of interest**

The authors declare that they have no conflicts of interest.

**References**


the upper respiratory tract and buccopharyngeal mucosa to nymphs of *Linguatula serrata*. Acta Trop 62: 127-134.


**Fig1.** SDS-PAGE analysis of somatic extracts (S), excretory- secretory products (ES) of *L. serrata*. M: molecular weight marker.
Fig 2. Immunoblot analysis of somatic and excretory-secretory antigens of *L. serrata* against a pool of sera from sheep infected with *L. serrata*. M: molecular weight marker; S (+): anti-*L. serrata* somatic antigen; ES (+): anti-*L. serrata* excretory-secretory antigen; S (-) and ES (-): negative serum.