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Original Article

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 present study aimed to analyze the content of crude somatic extracts and excretory-secretory products of *L. serrata* nymphs to detect the immune response of sheep and immunogenic proteins of the parasite. After collecting the nymphs, somatic extracts were prepared by sonication. Excretory secretory products were prepared by the incubation of nymphs in RPMI medium at 37°C with 5% CO2. Somatic and excretory-secretory proteins were isolated using SDS-PAGE. The 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 and excretory-secretory products 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, somatic extracts and excretory-secretory products demonstrated 12 and 3 antigenic proteins with molecular weights mostly in the range of 24-100 kDa and an antigen more than 180 kDa. Three common immunodominant antigenic proteins with molecular weights of 38 and 57, as well as an antigen of more than 180 kDa, were detected in the somatic extracts and excretory-secretory products of *L. serrata* nymphs in sheep with visceral linguatulosis. These antigens can be considetered prime candidates for future serodiagnosis and immunoprotective studies of the parasite.

Keywords: Antigen, Immunoblotting, Linguatula serrata, Sheep

1. Introduction

Linguatula serrata a parasitic arthropod from Pentastomida phylum, the family Linguatulidae, is a zoonotic parasite with an indirect life cycle. Adults live in the nose of dogs (and rarely of men), the body length of males is about 2 cm, whereas female parasites have a length of 2 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 has been reported in certain parts of the world, especially the Middle East, Southeast Asia, Africa, and America (3-8). The prevalence of linguatulosis in human populations is unknown since infections can only be diagnosed in the case of rare ocular involvement (9, 10) and incidentally discovered at laparotomy or autopsy (4, 7, 11). Linguatulosis in humans has been reported in 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 as 76.5%, 38%, and 53% in stray dogs in Iran (16), India (3), and Turkey (17), respectively. The infected dogs usually lack specific clinical signs; therefore, linguatulosis in these animals is usually detected at the surgery or autopsy by the isolation of the parasite from some organs, such as the liver, lungs, and lymph nodes (18). The natural infection of *L. serrata* in all domestic ruminants and some carnivorous animals has been reported in different geographical parts of Iran (16, 19-26). The importance of antibody (Ab) or antigen (Ag) detection in serological methods is of utmost importance in the diagnosis of infections.

Moreover, serological assays could also allow the processing of multiple samples in a short time, which would be useful in health management. There is a paucity of information on excretory-secretory (ESP) and somatic (S) antigens of *L. serrata*. In light of the aforementioned study, the current study aimed to characterize *L. serrata* antigens, which may be used as a basis for future studies on the specific immunological diagnosis of visceral linguatulosis in sheep and other animals.

2. Materials and Methods

2.1. 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. The mesenteric lymph nodes of the 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 was considered positive serum. The collected nymphs were washed several times with tap water and then used for somatic extract or excretory-secretory products. Negative sera were prepared from indoor lambs.

2.2. Preparation of S and ES Proteins

The collected nymphs of the parasite were separately washed three 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), 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) was obtained from the culture *in vitro* of the parasite nymphs. A total of 250 nymphs were placed in a 75 cm^2 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. The 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 three times (3X) under nitrogen flow. Protein concentrations of the S and ESP filtrates were determined by the Bradford method and stored at -20°C until use.

2.3. SDS-PAGE and Immunoblotting

The 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). In brief, 60 µl of each sample was mixed with 30 µl of a non-reducing sample buffer [SDS (10%), Tris-HCl (1M; pH 6.8), glycerol (2%)], boiled for 5 min, centrifuged for 1 min, and supernatants were 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 h. The gel was stained for protein visualization with Coomassie blue R-250 (0.1% w/v).

For immunoblotting, the 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 h at 60 V. Following that, the transferred proteins were blocked with blocking solution (5% skim milk in PBS) at room temperature for 1 h.

The TNC 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 were confirmed by enzyme-linked immunosorbent assay (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 four times, and the better dilution was selected 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 h at 37ºC and in a mixture of H2O2-Chlornaphtol for 15 min.

3. Results

The protein concentrations of S and ESP were obtained at 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) (Figure 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 (Figure 1).

The 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. seratta*) 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 (Figure 2). The results demonstrated three main proteins (bands) in the ESP of *L. serrata* nymphs with apparent molecular weights of 38, 57, as well as one specific polypeptide band (like to S) with a molecular weight of higher than 180 kDa (Figure 2).

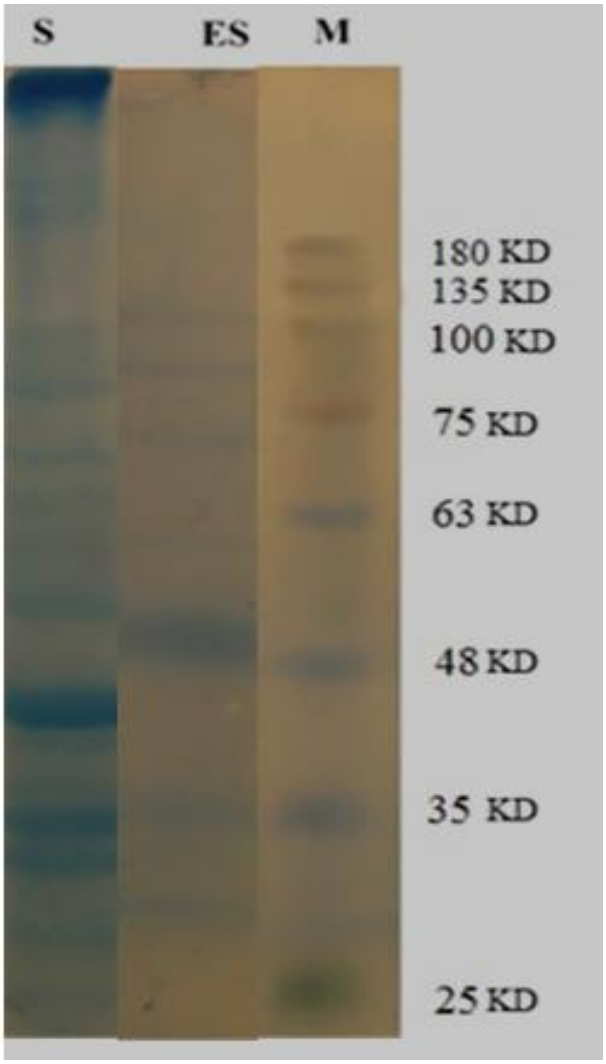

Figure 1. SDS-PAGE analysis of somatic extracts (S), excretory- secretory products (ES) of *L. serrata*. M: molecular weight marker

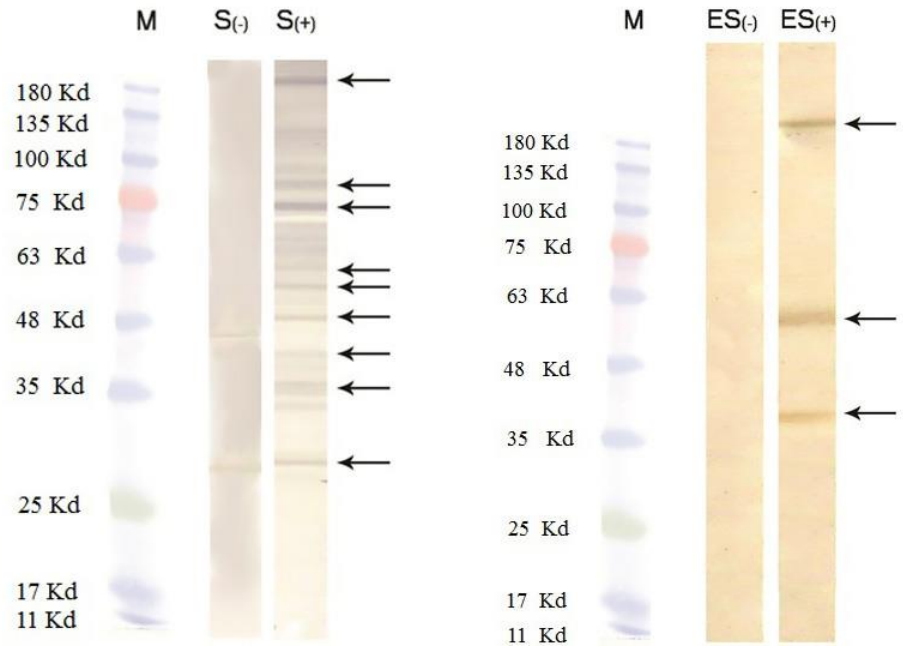

Figure 2. Immunoblot analysis of somatic and excretory-secretory antigens of *L. serrata* against a pool of sera from sheep infected with *L. serrata*. M: molecular weight marker; S (+): anti- *L. serrata* somatic antigen; ES (+): anti- *L. serrata* excretory-secretory antigen; S (-) and ES (-): negative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

4. Discussion

Linguatulosis in veterinary medicine has attracted assiduous attention in the countries across the globe, including Iran. The eggs of *L. serrata*, which contain larvae are ingested by different vertebrate intermediate hosts, such as ruminants and men (1, 2). There exist 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 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.* The results of the present study pointed to 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 by Hajipour, Tavassoli (28) is the only research available on the profile of somatic proteins of *L. serrata* nymphs. They demonstrated the presence of six 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). This discrepancy between the protein profiles of *L. serrata* nymphs in the stated study and those obtained in the current research can be ascribed to differences in methods and type of hosts (28).

In the present study, attempts were made to determine diagnostic antigens of *L. serrata*, which can be used for the specific immunological diagnosis of linguatulosis. Infection diagnosis 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 the serological diagnosis of infectious disease (29). Based on the results of the present study, immunoblot analysis of S and ES products using sera of ovine infected with *L. serrata* showed reactivity with 12 and 3 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, Ghorbanpoor (30) evaluated an ELISA test for the serodiagnosis of parasite infection in sheep by using S and ESP of *L. serrata*. Although in their study, the results of the sensitivity and specificity of the ELISA were almost the same for S and ES, ES antigens were better than S antigens for the detection of infection in sheep. The findings of both studies pointed to the similarity of antigens in the S and ESP of the parasite; moreover, they confirmed the common immunodominant antigenic proteins determined in the current study.

5. Conclusion

The results of the present study demonstrated three common immunodominant antigenic proteins with molecular weights of 38 and 57, as well as an antigen of more than 180 kDa, in the S and ESP of *L. serrata* nymphs in sheep with visceral linguatulosisthree. These antigens can be considered prime candidates for future serodiagnosis and immunoprotective studies of the parasite.

Authors' Contribution

Study concept and design: A. R. A.
Acquisition of data: S. B.
Analysis and interpretation of data: F. Kh.
Drafting of the manuscript: A. R. A.
Critical revision of the manuscript for important intellectual content: S. B.
Statistical analysis: F. Kh.
Administrative, technical, and material support: A. R. A.

Ethics

All procedures were approved by the ethics committee of the Shahid Chamran University of Ahvaz, Ahvaz, Iran under project number 2020-75487-45.

Conflict of Interest

The authors declare that they have no conflict of interest.

Acknowledgment

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References

1. Mehlhorn H. Encyclopedic Reference of Parasitology. 2nd ed: Heidelberg: Springer-Verlag; 2007.
2. Soulsby EJL. Helminths, Arthropods and Protozoa of Domesticated Animals: Lea & Febiger; 1982.
3. Acha P, Szyfres B. Pentastomiases. zoonosis and communicable diseases common to man and animals. 3. 3rd ed: PAHO; 2003. p. 345-50.
4. Baird JK, Kassebaum LJ, Ludwig GK. Hepatic granuloma in a man from North America caused by a nymph of Linguatula serrata. Pathology. 1988;20(2):198-9.
5. El-Hassan AM, Eltoum IA, El-Asha BMA. The Marrara syndrome: isolation of Linguatula serrata nymphs from a patient and the viscera of goats. Trans R Soc Trop Med Hyg. 1991;85(2):309-.
6. Lazo RF, Hidalgo E, Lazo JE, Bermeo A, Llaguno M, Murillo J, et al. Ocular linguatuliasis in Ecuador: case report and morphometric study of the larva of Linguatula serrata. Am J Trop Med Hyg. 1999;60(3):405-9.
7. Tappe D, Winzer R, Buttner DW, Strobel P, Stich A, Klinker H, et al. Linguatuliasis in Germany. Emerg Infect Dis. 2006;12(6):1034-6.
8. Yagi H, El Bahari S, Mohamed HA, Ahmed ERS, Mustafa B, Mahmoud M, et al. The Marrara syndrome: a hypersensitivity reaction of the upper respiratory tract and buccopharyngeal mucosa to nymphs of Linguatula serrata. Acta Tropica. 1996;62(3):127-34.
9. Lang Y, Garzozi H, Epstein Z, Barkay S, Gold D, Lengy J. Intraocular pentastomiasis causing unilateral glaucoma. Br J Ophthalmol. 1987;71(5):391-5.
10. Riley J. The Biology of Pentastomids. In: Baker JR, Muller R, editors. Advances in Parasitology. 25: Academic Press; 1986. p. 45-128.
11. Ma KC, Qiu MH, Rong YL. Pathological differentiation of suspected cases of pentastomiasis in China. Trop Med Int Health. 2002;7(2):166-77.
12. Anaraki Mohammadi G, Mobedi I, Ariaiepour M, Pourmohammadi Z, Zare Bidaki M. A Case Report of

Nasopharyngeal Linguatuliasis in Tehran, Iran and Characterization of the Isolated Linguatula serrata. Iran J Parasitol. 1970;3(1).
13. Maleky F. A case report of Linguatula serrata in human throat from Tehran, central Iran. Indian journal of medical sciences. 2001;55(8):439-41.
14. Sadjjadi S, Ardehali S, Shojaei A. A Case Report Of Linguatula Serrata In Human Pharynx From Shiraz, Southern Iran. Med J Islam Repub Iran. 1998;12(2):193-4.
15. Siavashi M, Assmar M, Vatankhah A. Nasopharyngeal Pentastomiasis (Halzoun): Report of 3 Cases. Iran J Med Sci. 2015;27(4):191-2.
16. Alborzi A, Molayan PH, Akbari M. Prevalence of Linguatula serrata Nymphs in Mesenteric Lymph Nodes of Cattle and Buffaloes Slaughtered in Ahvaz Abattoir, Iran. Iran J Parasitol. 2013;8(2):327-32.
17. Tasan E. Distribution of Linguatula serrata (Frohlich, 1789) in dogs from rural districts of Elazığ. Doğa Vet Hay Derg. 1987;11:86-9.
18. Oluwasina OS, Thankgod OE, Augustine OO, Gimba FI. Linguatula serrata (Porocephalida: Linguatulidae) Infection among Client-Owned Dogs in Jalingo, North Eastern Nigeria: Prevalence and Public Health Implications. J Parasitol Res. 2014;2014:916120.
19. Alborzi A, Darakhshandeh T. A survey on infection of Linguatula serrata nymphs in sheep slaughtered at Yasuj abattoir. Iran J Vet Res. 2007;4:103-9.
20. Esmaeilzadeh S, Mohammadian B, Rezaie A. Linguatula serrata nymph in a cat. Iran J Vet Res. 2008;9:387.
21. Nourollahi Fard SR, Kheirandish R, Asl EN, Fathi S. Mesenteric and mediastinal lymph node infection with Linguatula serrata nymphs in sheep slaughtered in Kerman slaughterhouse, southeast Iran. Trop Anim Health Prod. 2011;43(1):1-3.
22. Oryan A, Khordadmehr M, Ranjbar VR. Prevalence, biology, pathology, and public health importance of linguatulosis of camel in Iran. Trop Anim Health Prod. 2011;43(6):1225-31.
23. Oryan A, Sadjjadi SM, Mehrabani D, Rezaei M. The status of Linguatula serrata infection of stray dogs in Shiraz, Iran. Comp Clin Path. 2008;17(1):55-60.
24. Razavi SM, Shekarforoush SS, Izadi M. Prevalence of Linguatula serrata nymphs in goats in Shiraz, Iran. Small Rumin Res. 2004;54(3):213-7.
25. Saiyari M, Mohammadian B, Sharma RN. Linguatula serrata (Frolich 1789) nymphs in lungs of goats in iran. Trop Anim Health Prod. 1996;28(4):312-4.
26. Shekarforoush SS, Razavi SM, Izadi M. Prevalence of Linguatula serrata nymphs in sheep in Shiraz, Iran. Small Rumin Res. 2004;52(1):99-101.
27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5.
28. Hajipour N, Tavassoli M, Tayefi-Nasrabadi H, Seyedi S. Comparative evaluation of somatic proteins of the nymphal stage of Linguatula serrata isolated from goats and cattle in northwest Iran. Bulg J Vet Med. 2016;19:242-6.
29. Ogunremi O, Lankester M, Loran S, Gajadhar A. Evaluation of excretory-secretory products and somatic worm antigens for the serodiagnosis of experimental Parelaphostrongylus tenuis infection in white-tailed deer. J Vet Diagn Invest. 1999;11(6):515-21.
30. Alborzi AR, Ghorbanpoor M, Hamidnejat H, Poormehdi Boroujeni M, Mehdizadeh A. Development And Evaluation Of An Indirect Elisa Test For Serodiagnosis Of Linguatula Serrata Infection In Sheep. Iran J Vet Res. 2015;11(1).