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Original Article

An Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to *Linguatula serrata* in Experimentally Infected Dogs

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Abstract

Background: *Linguatula serrata* is a causative agent of visceral and nasopharyngeal linguatulosis in humans and animals. The aim of the present study was to investigate the immune response of dogs experimentally infected by *L. serrata* with ELISA.

Methods: Five puppies were infected by inserting the *L. serrata* nymphs in their nasal cavities (infected group) in the Department of Parasitology of Shahid Chamran University of Ahvaz, during 2018-2019. Three animals were kept as the non-infected control group. Blood samples were collected from the animals for seven months at approximately monthly intervals for serum preparation. Nasal samples were taken weekly from the fourth month. ELISA was designed and performed on 64 sera (24 negatives, and 40 positives) using somatic (S), and excretory-secretory (ES) antigens.

Results: Overall, 100% of the animals were infected with the parasite. Based on the results of ELISA, the ES antigen (sensitivity 95% and specificity 92%) was more preferred than the S antigen (sensitivity 95% and specificity 85%). Female parasites had significant effects on the immune response. There was a significant correlation between the clinical symptoms and the presence of female parasites ($P<0.05$).

Conclusions: The results showed a practical method for dogs' experimental infection. ELISA method is suitable for the detection of infection at different stages of development, especially before the maturation stage of the parasite. In this regard, the ES antigen of the parasite was more immunogenic. Therefore, ELISA can be used as a serological method in the early detection and epidemiological studies of infection with *L. serrata* in dogs.

Introduction

Linguatula serrata is a zoonotic parasite with adult parasites living in the respiratory and nasal passages of dogs, other canines and, rarely humans. Humans and herbivores are infected by ingesting the parasite eggs. Parasite nymphs migrate to various organs (e.g., mesenteric lymph nodes, liver, and lungs) and become infectious. The definitive host develops nasopharyngeal linguatulosis by ingesting the viscera of animals infected with the nymphs, which migrate to nasal passages after 6-7 months. Humans may be infected with nymphs called Halzoun syndrome or Marrara syndrome that is an immediate hypersensitivity or allergic reaction to the pentastomid. Its inflammatory effects are usually restricted to the tissues of the throat, larynx, tonsils, nasal passages, and lips. Occasionally, the affected person may die due to suffocation during the first few hours after onset. Infection with the visceral form (visceral linguatulosis) in the intermediate hosts also results in lesions in the organs involved (1,2).

Surveys carried out by main host autopsy in Iran revealed that 62.2% of stray dogs in Shahrekord (3), 76.24% of Shiraz dogs (4), 53.2% of Marand dogs (5), 40.62% of Ilam dogs (6), and 27.83% of dogs in the northwest of Iran were infected (7). Although dogs' infection may sometimes be accompanied by clinical symptoms of sneezing and nasal discharge, these manifestations are not specific and cannot be detected because of the long pre-puberty stage of the parasite (8). Therefore, investigation of serological methods that can detect infected animals, especially in the early stages (before maturation of the parasite); will be of great importance.

The aim of the present study was to investigate the long-term-humoral immune response of dogs experimentally infected by *L. serrata* with ELISA.

Materials & Methods

Ethical consideration

This research has been approved by the Ethics Committee on Animal Research and Animal Rights of the Shahid Chamran University of Ahvaz, with code EE / 97.24.3.70440 / scu.ac.ir.

Experimental infection

Mesenteric lymph node of 400 native sheep was examined for the presence of the nymphal stage of *L. serrata* between Jan to July 2018. After slaughtering, the visceral organs were taken out and one to 10 mesenteric lymph nodes were collected from each sheep. The sampled lymph nodes were placed in separate pots containing normal saline and were transferred to the Parasitology Laboratory of the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran. Each lymph node was cut longitudinally, put in Petri dishes containing normal saline, and examined under a dissecting microscope for *L. serrata* nymphs.

Eight puppies -2 to 3-month-old- that were monitored from birth and were free of *L. serrata* were selected for the experimental trial. Puppies were housed in covered pens and fed daily with laboratory chow throughout the experiment.

To induce experimental infection five *L. serrata* nymphs were transferred with a plastic pipette to the right and left nasal cavities of five puppies (2 females and 3 males) (infected group). Nymphs were transferred in one or two serum saline droplets so that they would not have sneezed out. In addition, three puppies (males) were kept uninfected as a control group for seven months and fed with laboratory chow throughout the experiment.

Blood and nasal sampling

Blood samples were taken from animals (5 infected and 3 uninfected groups) about 1 month before inoculation, and for 7 months after infection at intervals of approximately monthly (8 times) (n= 64). Ketamine (15 mg/kg) and acepromazine (0.15 mg/kg) were injected for sedative effects. Blood samples were collected from a cephalic or saphenous vein in test tubes without anticoagulant and allowed to clot, and then centrifuged for 10 min at 2400 × g. The sera were separated and kept in 1.5 ml microtubes at -30 °C.

From the fourth month onwards, nasal samples from the infected animals were taken every week with swabs and examined by light microscopy for *L. serrata* eggs. During the infection period, the animal's clinical symptoms were also considered.

After microscopic observation of the first egg, dogs were euthanized intravenously using thiopental sodium (50 mg/kg) and examined by nasal and frontal sinus autopsy. The parasites were counted and transferred to physiological serum or 70% alcohol. The conversion index of nymphs to adults of the parasite (CI n.a) was calculated as the following formula.

CI n.a= N/A x 100

CI n.a = Conversion index of nymphs to adults

N=number of nymphs transferred to the nasal cavity

A= number of adults obtained

Preparation of the somatic (S) and excretory-secretory (ES) antigens (Ags) of L. serrata nymph

Nymphs of *L. serrata* were collected from the mesenteric lymph nodes of infected animals. Preparation of the S and ES antigens was carried out according to Alborzi et al., (2015) study (18). After the determination of protein concentration by the Bradford method antigens were distributed in sterile microtubes and kept at -30 °C.

Development and optimization of ELISA

The optimum dilution of antigen for coating and the best dilution of serum and conjugate in ELISA were determined separately for each of the antigens by cross-table (checkerboard) titration method. Five dilutions (1: 50, 1: 100, 1: 250, 1: 500, and 1: 1000) for S antigen with 200 µg/ml protein were evaluated. ES antigen was evaluated according to its protein content (100 µg/ml) in five dilutions (i.e.: 1:10, 1:20, 1:40, 1:80 and 1:160). For dog anti-IgG conjugate different concentration (1: 1000, 1: 2000, 1: 4000, 1: 8000, 1: 16000) were tested. Serum was also diluted in 3 dilutions (1:50, 1: 100, and 1: 200).

Using the best dilutions of S and ES antigens, serum and conjugate, as well as positive and negative control serum, ELISA was performed as follow:

Sixty-four serum samples (24 negatives and 40 positives) from the animals were examined.

The 96-well plates (Nunc, Denmark) were coated with 100 µL of 1:40 dilution of ES antigen, refrigerated overnight (4 °C), and then washed twice with 0.1% PBST. For blocking 200 µL of 2% PBST was added to the wells and washed 2 hours later (at laboratory temperature) just as in the previous section.

Optimal serum dilution (1:25) was added to the plate. After 2 hours at room temperature, they were washed 4 times with 0.1% PBST. Then 100 µl of 1: 8000 conjugate dilution (Abcam, UK) with 0.1 PBST was added to each plate and incubated for 1 hour at laboratory temperature. The solution was washed similar to the previous section. Finally, 100 µl of chromogenic substrate solution (TMB) (Rahazistpadtan, Iran) was added to the wells, and after 15 min, 100 µl of reaction stop solution, 2N sulfuric acid (Merck, Germany), was added to the wells. The results were recorded by an ELISA reader as optical density at 450 nm.

The optical density results of the samples (positive, n= 40, and negative, n=24) in different periods were obtained and S/P percentage for each sample was determined.

After determining the S/P percentages of the samples, the results were adjusted over approximately two months' periods (T1, T2, T3, and T4).

Using the MedCalc statistical software version 19.1.3, by drawing a rock curve and determining the optimal cut-off point, ELISA indices were determined for both antigens.

Statistical Analysis

Statistical analysis of the data was performed using SPSS software version 16 (Chicago, IL, USA) and normal distribution test (Kolmogorov-Smirnov), repeated measures, paired *t*-test, independent *t*-test, Spearman correlation, and Fei-Wei-Kramer correlation (with 95% confidence interval). A *P*-value of less than 0.05 was considered significant.

Results

Experimental infection

Experimental infection in five dogs showed that all of them (100%) were infected and had adult parasites. Four of them (80%) had adult male and female parasites simultaneously, and in one dog (20%), only adult male parasites were obtained. As shown in Table 1, from the 50 nymphs that were placed in the nasal cavity of the five dogs in the infected group, 24 adult parasites (10 males and 14 females) were obtained. These results indicate that in this method, 48% of the nymphs were able to attach to the mucous membrane in the nasal cavity, survived, and became adults. Therefore, 20% and 28% of the nymphs became males and females, respectively. In total, the conversion of nymphs to adult parasites (CI n.a) in the infected group was between 20% and 100%, and among the isolated adult parasites, 58.3% and 41.6% were male and female, respectively.

Table 1: Results of the experimental infection of dogs with *Linguatula serrata* nymphs

Dog No.	***Nasal cavity***	***Nymphs No.***	***Adult parasite obtained***						***CI n.a***	***Parasite eggs***	***Clinical sign***
			Female		Male		Total (F+M)				
			%	No.	%	No.	%	No.			
	right	5	28.6	2	0	0	28.6	2	40		
1	left	5	28.6	2	42.9	3	71.4	5	100	+	+
	total	10	57.1	4	42.9	3	100	7	70		
	right	5	50	2	25	1	75	3	60		
2	left	5	25	1	0	0	25	1	20	+	+
	total	10	75	3	25	1	100	4	40		
	right	5	0	0	25	1	25	1	20		
3	left	5	0	0	75	3	75	3	60	-	-
	total	10	0	0	100	4	100	4	40		
	right	5	0	0	25	1	25	1	20		
4	left	5	75	3	0	0	75	3	60	+	+
	total	10	75	3	25	1	100	4	40		
	right	5	60	3	0	0	60	3	60		
5	left	5	20	1	20	1	40	2	40	+	+
	total	10	80	4	20	1	100	5	50		
		25	50	7	30	3	41.7	10	40		
Total		25	50	7	70	7	58.3	14	56	------	------
		50	28	14	20	10	48	24	48		

CI n.a: Conversion index of nymphs to adults

Clinical symptoms were also observed in the T4 period in the infected group. These general symptoms included sneezing, difficulty breathing, tremors of the nose, scratching the nose,

and in some of them, increased nasal discharge and nervous symptoms. However, no clear clinical signs were observed neither in the control group nor in the infected group before the T4 period.

ELISA results

Appropriate dilutions of antigens, dog anti-IgG conjugate, and serum were determined for the diagnosis of *L. serrata* infection in dogs by ELISA (Table 2). This experiment was conducted for more than 7 months and the results were adjusted in 4 periods (T1: 1-54 days post-infection (dpi); T2: 55-108 dpi; T3: 109-162 dpi; T4: 163-216 dpi.). ES antigen (with 95% sensitivity and 92% specificity) was more appropriate than S antigen (with 95% sensitivity and 85% specificity) for detection of *L. serrata* infection (Fig. 1, 2 and Table 3).

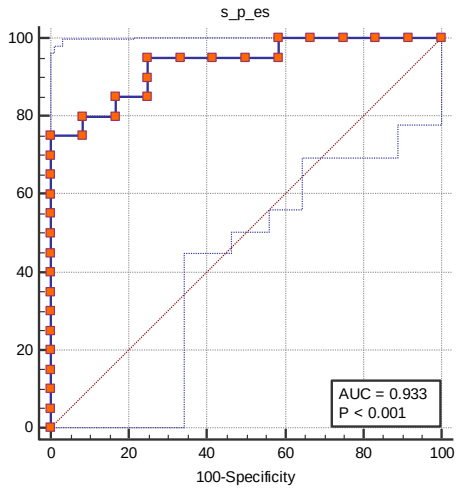

Fig. 1: Mean sensitivity and specificity of ELISA with ES antigen of *L. serrata* for 7 months in infected dogs

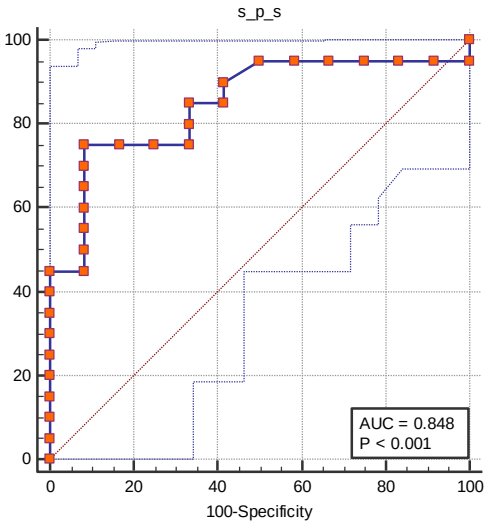

Fig. 2: Mean sensitivity and specificity of ELISA with S antigen of *L. serrata* for 7 months in infected dogs

Table 2: Results of appropriate dilutions of antigens, dog anti-IgG conjugate, and serum determined for the diagnosis of *L. serrata* infection in dogs by ELISA

Antigen type	*Anti IgG conjugate dilution*	*Serum dilution*	*Ag dilution*
S	1:8000	1:50	1:500 (0.4µg/ml)
ES	1:8000	1:50	1:40 (2.5µg/ml)

Table 3: ELISA Indices for diagnosis of *L. serrata* at different stages of experimental infection.

Period	*Antigen type*	*Specificity(%)*	*Sensitivity(%)*	*NPV(%)*	*PPV(%)*
T1	ES	100	80	75	100
	S	66.67	80	50	75
T2	ES	66.67	100	100	83.3
	S	100	100	100	100
T3	ES	100	100	100	100
	S	100	100	100	100
T4	ES	100	100	100	100
	S	33.33	100	100	71.4
Total	ES	91.66	95	93.75	95.82
period	S	75	95	87.5	86.6

NPV: Negative Predictive Value; PPV: Positive Predictive Value

Based on the Kolmogorov-Smirnov test, the statistical distribution of control and infected group data was normal. Repeated measures test results showed a significant difference between the infected and control groups using S and ES antigens at different sampling periods (T0-T4; *P*= 0.041). The statistical comparison of consecutive periods in the experimental group for ES antigen showed an increasing trend between T0 to T3. The decreasing trend in the T3 to T4 interval was not significant. The analyses of the control group showed a nearly uniform trend and showed no significant changes (*P*= 0.739; Fig. 2). Somatic antigens in the infected group had an increasing trend between T0 and T1 as well as T1 and T2. After that, until T4, a decreasing trend was noted, but this decrease was not significant (*P*= 0.140). In the control group, there were slight reductions in S antigen over the periods, none of which were significant (*P*= 0.912).

Statistical comparison (*t*-test) of the mean of the infected and control groups in the sampling periods with ES antigen showed a significant difference in all periods (*P*= 0.032). As expected, time T0 showed no significant difference between experimental and control groups (*P*= 0.847). However, this comparison was significant (*P*= 0.021) when using S antigen, especially in T2 and T3. Spearman correlation test results showed that female parasites at different periods, especially T2 and T3, had a significant effect on the immune response to ES antigen and in T2 to the S antigen. There was no significant relationship between male parasites in the nasal passages of infected animals and immune response to both S and ES antigens. In other words, the male parasites had no significant effect on the immune response (*P*= 0.450).

There was a significant correlation between the clinical symptoms (in the T4 period) and the presence of female parasites (*P*= 0.014). This means that the presence of female parasites plays an essential role in the onset of symptoms. No such relationship was observed with male parasites (*P*= 0.447).

Discussion

L. serrata is of great medical and veterinary importance (9,10). Infection with this parasite has been reported in dogs in some parts of Iran and around the world by autopsy or nasal secretion testing for parasite egg observation (3, 4, 6, 7, 11-15). Diagnosis of linguatulosis is often difficult due to the lack of specific clinical indications in infected dogs (14).

The results of the present study showed that in experimental infection, 100% of the animals were infected with the parasite. While 80% of the infected dogs had adult male and female parasites simultaneously, in 20% only adult male parasites were detected. In infected animals whose only male parasites are present, conventional laboratory methods based on the parasite egg observation cannot be applicable. This can be regarded as an occult infection, which is not detectable by the common parasitological methods. Therefore, designing other methods should be considered.

Regarding the effects of this parasite on humans and animals, its early detection in dogs can be very effective in controlling the infections. In the present study, an ELISA test was designed for the detection of *L. serrata* and investigating dogs' humoral immune response during the 7 months. The ascending humoral immune response to the S and ES antigens of the parasite was detected until 3-4 months (T2) and 6-5 months (T3) after infection, respectively. The ES antigen had a better performance (sensitivity and specificity) than the S antigen for detecting the *L. serrata* infection during the four periods after infection. Based on the location of the parasites in the nasal cavities and the size of the parasites, it seems that the ES products are more exposed to the immune system. The examination of feces in dogs showed positivity in 25 samples, while the indirect ELISA test of dogs' sera showed seropositivity in 30 samples (16). In their study, the indirect ELISA test of dogs' sera showed sensitivity of 84% and specificity of 64%, with an accuracy of 74% (16). It should be mentioned that in their study examination of feces was chosen as golden standard test. In their study, serological tests were designed based on dogs that were naturally infected to the adult stages of *L. serrata* while in our study alterations in immune response were investigated in experimentally infected dogs from early infection to the adult stage of the parasite.

Since there is no available information about the immunology of *L. serrata*, therefore, based on its similarity to *Oestrus ovis* the results of the present study were mainly compared with studies of sheep nasal bot flies.

The humoral response of sheep and goats to *O. ovis* was associated with immunoglobulin production locally or systemically (17). The ES antigens were better than the S antigens in the ELISA test to detect *L. serrata* infection in sheep (18).

Serum IgG antibody changes in repeated animal inoculation experiments with *O. ovis* usually peak 2 to 4 weeks after the first infection (19). However, in the present study, these changes were delayed and increased about 4-8 weeks after infection, which may be due to differences in the growth rate, evolutionary and physiological changes of *L. serrata* compared to *O. ovis*, which lives only some part of its evolutionary phase in the host.

The results showed that the number of males had no significant effect on the immune response. This may be justified by the small size of males (up to 2 cm) compared to the larger females (about 12 cm). *O. ovis* first stage larvae have a low stimulating effect on the animal's immune system due to their smaller size and lower nutritional requirements (20). The humoral immune response of sheep and goats to *O. ovis* has shown that the IgG level is strongly enhanced by larval development to the second and third stages (17, 21-24).

In addition, the number of female *L. serrata* was more effective on the immune response to ES and S antigens at T1-T2 and T2 periods,

respectively, because the higher number of parasites may produce more available ES antigens to the immune system.

It can be concluded that females play a major role in the production of ES antigens. Possibly, at the T2 period, certain physiological changes occur along with an increase in the number of females that increase ES antigen production. Therefore, these results claim that the female parasite, its ES products, and the evolutionary stage of the parasite (the age of the parasite) affect the host immune response.

The symptoms of the parasite mainly develop in the late developmental stage, especially with the presence of adult female parasites. It may be due to the size, spines of the body surface, egg production, stimulatory and obstructive effects of females on the nasal passage, and mucosa. Since, the symptoms of this parasite are not entirely specific and can be caused by other factors (viral, bacterial, etc.) therefore, this infection may be neglected (8, 25).

Conclusion

Inserting the *L. serrata* nymphs into the nasal cavities could be an appropriate method for experimental infection in dogs. In addition, the ELISA method could be used to detect the infection in dogs in the pre-adult stages of the parasite. Detection of the infection before maturation in the main host (dogs) and their treatment can prevent the infection of intermediate hosts including ruminants. This will play an important role in the epidemiology of the parasite, controlling and preventing the spread of linguatulosis in animals, and especially in humans.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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