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DIAGNOSIS OF CYSTIC HYDATIDOSIS IN EXPERIMENTALLY INFECTED SHEEP BY ELISA

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ABSTRACT

Aim: Cystic echinococcosis, a zoonotic infection, is caused by metacestod formation of the *Echinococcus granulosus,* and it causes major economical loses and health disorders. This study was carried out to determine the sensitivities of ELISA test in order to serologic diagnosis of cystic echinococcosis in experimentally infected sheep.
Materials and Methods: A total of 20 Akkaraman sheep (4-5 months) were subjected in this study as 10 for control and another 10 for experimental group. A total of 15.000 live protoscoleces collected from liver with hydatid cyst were administered into a dog via oral route, and at the end of two months, maturated *E. granulosus* was collected from intestinal lumen. Approximately 2000 eggs were administered into each sheep in the experiment group. Sheep had been observed for 12 months. During this period, all the sheep were checked by using ELISA for parasite specific antibodies.
Results: ELISA test used with partially purified cyst fluid antigen had shown 57% sensitivity and 67% specifity when compared to necropsy. Antibody responses in sheep during 12 months were shown different peaks in different times. The foremost antibody response was determined in two weeks after inoculation. No antibody response was determined in calcified cysts.
Conclusion: It may be stated that ELISA test are more reliable for the diagnosis of cystic echinococcosis, and necropsy findings can be used as reference for determination of sensitivity and specifity of serology.

Keywords: ELISA, cystic hydatidosis, sheep.

INTRODUCTION

Cystic echinococcosis, a zoonotic infection, is caused by metacestod formation of the *Echinococcus granulosus,* and it causes major economical loses and health disorders. The life cycle includes two mammalian hosts. The definitive hosts are carnivores such as dogs and wolves. Intermediate hosts are domestic mammals and humans. The definitive hosts take protoscoleces from the cysts, while humans and other intermediate hosts are infected by taking the eggs from definitive hosts' feces. The diagnosis of the disease in intermediate host animals is of great importance for the success of the control programs for Echinococcus infections (Senlik, 2004). Compared to humans, the number of studies conducted for the purpose of immunological diagnosis of hydatidosis in intermediate hosts is limited. Complement fixation

assays, agglutination assays, double gel diffusion, immunoelectrophoresis, ELISA and western blot have been used in serologically diagnosed studies of sheep hydatidosis (Sweatman et al. 1963; Blundell-Hasell, 1969; Conder et al. 1980). In the studies conducted for the purpose of immunodiagnosis of hydatidosis, ELISA in which various antigens are used can be used for diagnosis of flock-based infection but it is stated that individual animal-based diagnosis is not reliable (Lightowlers et al. 1984; Yong et al. 1984; Kittelberger et al. 2002).

This study was planned to determine the sensitivity of the ELISA in the serological diagnosis of hydatidosis in experimentally infected sheep and to determine if it is possible to detect the disease early by this test.

MATERIALS AND METHODS

In this study, a total of 20 male Akkaraman lambs at 4-5 month of ages were used for experimentally study. Animals were housed in the Veterinary Faculty during experimentally period. The lambs were divided into 10 experimental group and 10 as the control group. In sterile conditions, the cyst fluid was aspirated with the injector and the protoscoleces were separated from the sheep liver and the viability of the protoscoleces was determined by Methylene blue staining. A total of 15.000 live protoscoleces collected from the liver with hydatic cyst were administered into a dog via oral route and at the end of two months, maturated *E. granulosus* adults were collected from the intestinal lumen after euthanasia. Collected adults were washed in physiological saline and mature proglottids were crushed and approximately 2000 eggs were administered into each sheep in the experiment group. They were followed up for 12 months. Sufficient blood samples were collected weekly for two months after the eggs were administered orally, and then continued to be collected monthly until necropsy. After centrifugation, sera samples were separated and stored at -20^{0}C until used. The samples were tested by ELISA. At the end of 12 months, all sheep were necropsied and all organs and tissues, especially liver and lungs, were investigated macroscopically for hydatid cyst.

Preparation of partial purifiy cyst fluid antigen (AgB): AgB was prepared from hydatic cyst fluid derived from sheep liver by procedures described else-where. Briefly, the hydatid cyst fluid obtained from the sheep liver was centrifuged at 5000 g for 10 minutes to allow precipitation of protoscoleces and other particles. This supernatant is dialyzed against 0,005 M acetate buffer (pH = 5.0) at +4℃ for 1 night and then centrifuged at 15000 g for 30 minutes at +4℃. The supernatant pellet was collected and dissolved in 10 ml of 0.2 M phosphate buffer (pH = 8.0). The pellet is discarded, allowing the solution to be stored for 15 minutes in a water bath and then at 1 hour at 20000 g at +4 DEG C. to obtain a supernatant rich in Antigen B (Simsek et al. 2006).

ELISA: The ELISA test was carried out according to the procedure described by Simsek et al. Serum samples and conjugate were diluted 1/200 and 1/5000, respectively. Results were taken as absorbance value. The standard deviation of the absorbance values of the negative control sera tested was multiplied by 2, and the positivity limit (cut-off) was determined by means of an arithmetic mean. Values above this value are considered as positive. Standard deviation was calculated by SPSS 23.0 statistical program. Necropsy and ELISA results were evaluated by chi-square test. P = .05 was regarded as statistically significant.

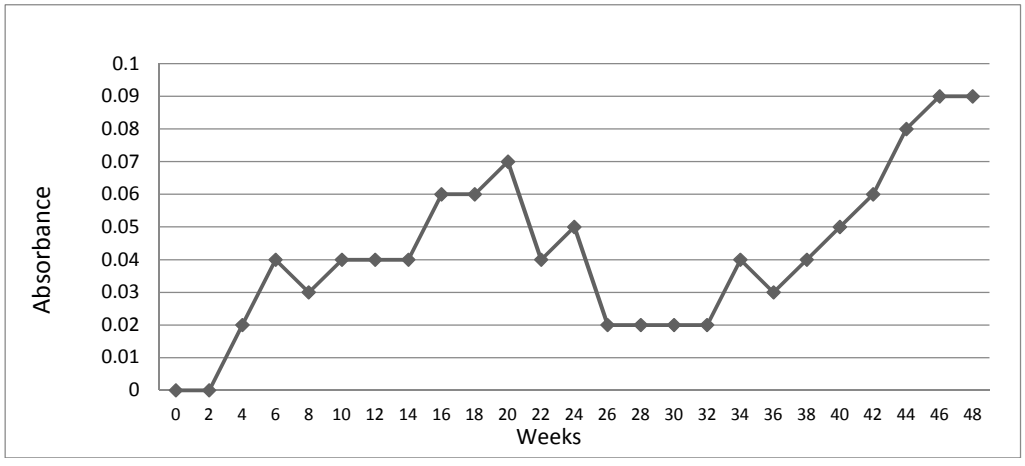
RESULTS AND DISCUSSION

Necropsy was performed at 12 months after experimental infection and all organs and tissues including liver and lungs were examined macroscopically (Fig. 1) and histopathologically for hydatid cyst (Fig. 2). The macroscopic examination findings are shown in Table 1.

Antibody responses formed in infected sheep were indicated in Graphic 1. Antibody response peaked at different times and the earliest antibody response was detected two weeks after inoculation. No antibody response was detected in cysts that formed calcification. In this study, the necropsy results were considered as the gold standard and the sensitivity and specificity of the ELISA test and the compatibility between the two data were calculated (Table 2). The sensitivity and specificity rates of the ELISA test used in the study were determined according to the criteria specified in Table 2. With this study, the specificity of ELISA was found to be 66.6% and the sensitivity to be 57.1%. The false negative rate was 42.8% and false positivity rate was 33.3%. There was no statistically significant difference between necropsy findings (7/10, 70%) and serological findings (4/10, 40%) ($P > 0.178$).

Table 1. Necropsy results of the experimental group

Animal number	Liver		Lung		Total number of cysts
	Number of cysts	Cyst diameter (cm)	Number of cysts	Cyst diameter (cm)	
1	2	0.1	2	0.7-0.5	4
2	3	0.1	4	0.7-0.5	7
3	2	1.5	-	-	2
4	-	-	3	0.5-1	3
5	-	-	-	-	
6	-	-	-	-	
7	-	-	-	-	
8	1	0.1	-	-	1
9	2	0.2-0.7	-	-	2
10	1	0.1	-	-	1
Total	11		9		20



Graphic 1. The average of antibody response in the experimental group

Table 2. Comparison of ELISA and necropsy results in the experimental group

		Necropsy		
ELISA		+	-	Total
	+	4 (a)	1 (b)	5
	-	3 (c)	2 (d)	5
	Total	7	3	10

Sensitivity; a/a+cX100, Specificity; d/b+dX100, False positivity rate; b/b+dX100, False negative rate; c/a+cX100

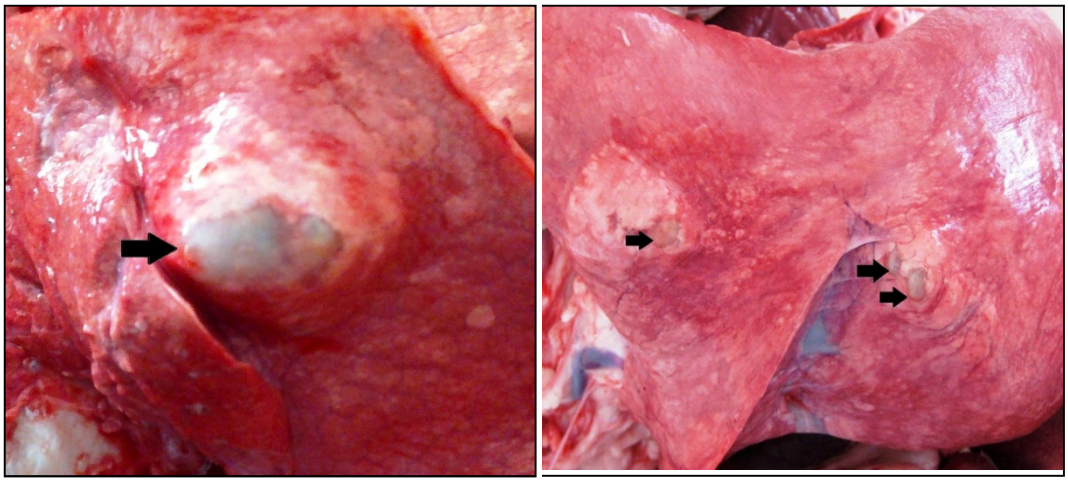


Fig. 1. Hydatid cyst in the sheep lung

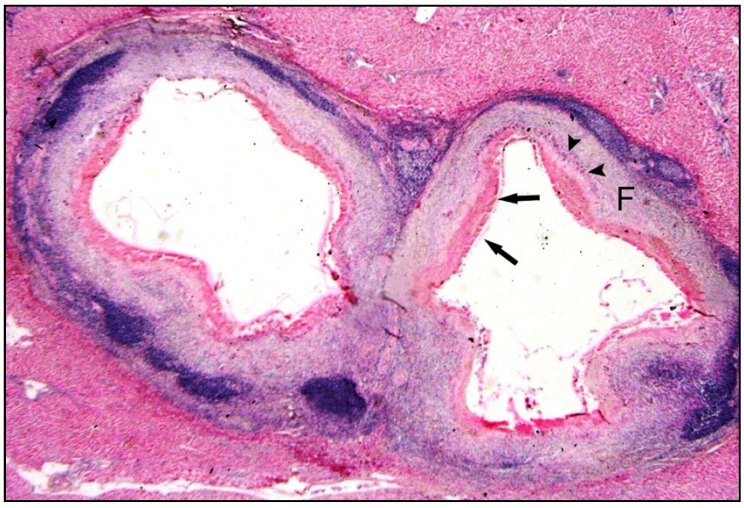


Fig. 2. Histopathological view of the cysts. Arrows: Cyst wall, Arrow heads: Foreign body giant cells, F: Fibrous capsule (H.E. X20)

Compared to humans, the number of studies conducted for the purpose of immunological diagnosis of hydatidosis in intermediate animals is very limited. Studies conducted for the purpose of immunodiagnosis of hydatidosis indicate that the ELISA test using various antigens may be used to diagnose of infection on a flock basis but it is not reliable on an individual animal basis (Lightowlers et al. 1984; Yong et al. 1984; Kittelberger et al. 2002). Ris et al. (1987) who performed ELISA using polysaccharide antigens obtained from in vitro cultured protoscolex and hydatid cyst membranes determined the specificity of the test as 81.8% and the sensitivity as 80.6%. Kittelberger et al. (2002) applied 8 kDa antigen (8 kDa ELISA) purified from hydatid cyst fluid and recombinant EG95 oncosphere protein (onc ELISA) and investigated the location of these antigens in sheep hydatidosis and found that the specificity of ProtELISA was 95.8-99.5%, and their sensitivity was 51.4-62.7%. Simsek and Koroglu (2004) determined the sensitivity and specificity of the ELISA using sheep crude cyst fluid antigen as 60% and 94%, respectively. Nijeruh and Gathuma (1987) found 91% sensitivity in naturally infected sheep and goats by ELISA method using antigen 880 (enriched with heat-stable antigen-B) purified from bovine hydatid cyst fluid. Ibrahem et al. (1996) obtained similar results (90% sensitivity) using purified AgB prepared from camel hydatid cyst fluid in the ELISA. Khan et al. (1990). reported the sensitivity of ELISA to be 42.9%, 60%, 62.5%, and specificity 80%, 60% and 0%, respectively, in the study conducted in bovine, buffalo and camel. In this study, partially purified cyst fluid antigen (AgB) was used in serologic diagnosis of sheep hydatidosis and the sensitivity of ELISA was 57.1% and the specificity was 66.6% (Table 2). In terms of sensitivity of ELISA, the ratio obtained in this study was almost the same as that reported by Kittelberger et al. (2002), but it was found to be lower than that reported by Ibrahem et al. (2002) and close to that reported by Simsek and Koroglu (2004). AgB has been used instead of crude cyst fluid antigen in this study because it provides more sensitive and specific responses in the literature.

No correlation was found between cyst number and antibody response in this study (P <0.310). Yong et al. (1984) performed an ELISA test in experimental infected sheep using the antigens prepared from sheep hydatid cyst fluid and obtained 32% sensitivity, 65% specificity. Reported that these antigens used in the ELISA were not suitable for the specific diagnosis of experimental and naturally infected sheep. The antigen used for sensitivity and efficiency of the test reports that the parasite should contain antigenic determinants against all antibodies produced by the animals at various stages of development. Ris et al. (1987) obtained 80% sensitivity and specificity in experimental infected sheep by ELISA using two different antigens. The sensitivity and specificity ratios found by Ris et al. (1987) are higher in this study, while the sensitivity rate found by Yong et al. (1984) is low and the specificity ratio is almost the same in this study. Lightowlers (1990) reported that there was no statistical correlation between the number of cysts and antibody titers. Blundell-Hasell (1969) reported that there was an increase in titers with the number of cysts, but no correlation between titers and number of cysts. Similarly, Yong et al. (1984) reported that the antibody response produced by the total number of cysts found in animals at the end of 12 months was not statistically significant. Senlik (2000) found a strong but not exact correlation between the number of cysts and antibody titers and

found that the IFAT and IHAT titers increased due to the increase in the number of cysts.

In this study, the earliest antibody response was detected within the second week after inoculation (Graphic 1). Lightowlers et al. (1984) reported that the ELISA caused a slow reaction during the first six months and eventually reached a high level after four years of infection in a two-month-old lamb that infected 2000 *E. granulosus* eggs orally. Lightowlers et al. (1986) reported that in their experimental study, sheep produced specific immunological responses to natural infection, but these responses did not lead to the formation of a continuous antibody. It also showed that antibody levels decreased despite the presence of parasite. Ghorbanpoor et al. (2006) reported that antibody response occurred after 8 weeks of infection in the IHA test where they were orally infected with 2000 *E. granulosus* eggs, and that the sensitivity of the test reached a maximum level of 11-12 weeks (92%) and then decreased. Ris et al. (1987) reported that antibody responses were elevated at 27 weeks post infection by ELISA in sheep infected with at least 100 *E. granulosus* eggs. Yong et al. (1984) reported that the earliest antibody response was detected within two weeks after infection, and that the antibody response detected with different antigens at 12 months peaked at different times. Larrieu et al. (2009) found the earliest immunologic response on the 10th day. The earliest antibody response in this study was close to that reported by Yong et al. (1984) and Larrieu et al. (2009).

CONCLUSION

As a result, the earliest antibody response could be detected two weeks after inoculation, the antibody response can not be determined in cysts formed by calcification and the ELISA test can be used because it is easy to administer and economical for the early detection of hydatidosis. However, new immunodominant antigens need to be investigated in order to increase the sensitivity and specificity of the test. It is also important to develop alternative diagnostic methods that can detect infection specifically in the early stages of infection.

ETHICAL APPROVAL

Research protocol was approved by SUVFEK (2009/68).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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