Immunization of dog with proteins under 30 kDa molecular weight of hydatid cyst fluid and protoscoleces of *Echinococcus granulosus*

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Abstract:

The aim of the present study was to assess the immunogenicity of under 30kDa molecular weight proteins of hydatid cyst fluid and protoscoleces in dogs experimentaly Infected with *Echinococcus granulosus*. Isolation of under 30 kDa proteins performed using Millipore filter. Six dogs were used in three groups: 2 dogs with under 30 kDa proteins of hydatid cyst fluid (Group I); 2 dogs with proteins of protoscoleces (Group II); and, 2 dogs were used as control groups (Group III). Two weeks later approximately 8,000 protoscoleces, with the viability of 80%, were given to each dog. On day 35 post infection adult worms were recovered from the intestines and the rate of infection determined by counting the total worms. The recovered worms in Group I were 197; in Group II were 207; and, in Group III were 382. The minimum number of adult worms (197) belonged to the dogs exposed to under 30 kDa molecular weight proteins of hydatid cysts fluid. This demonstrated significantly higher immunogenicity effects ($p<0.001$).

Introduction

Echinococcosis is a zoonotic disease caused by cestoda of the genus *Echinococcus* (Family *Taeniidae*). The species of major importance for public-health are *E. granulosus* and *E. multilocularis* which are the causing agents, respectively, of cystic echinococcosis and alveolar echinococcosis. Definitive hosts are carnivores such as dogs, wolves and foxes. Sexual maturity of adult *E. granulosus* occurs in the small intestine of final hosts within 5-6 weeks after the ingestion of offal containing viable protoscoleces (Zhang et al., 2006). Several studies have been carried out on the prevalence of infection in dogs from Iran. According to the rate of infection in intermediate and final hosts, Iran is an endemic region in the world (Eslami and Hosseini, 1998; Rokni, 2009). Dogs, as definitive hosts, are pivotal in the transmission of the hydatid cyst. Substantial efforts have been made to control of *E. granulosus* in the world, with limited success. Monthly treatment of dogs using praziquantel resulted in significant reduction in rates of *E. granulosus* infection in sheep (Zhang and McManus, 2008). Vaccination can provide an adjunct to improved, integrated control. Dog vaccination can provide an acceptable and cost-effective complementary control method, because there are far fewer dogs than sheep in areas where the parasite is endemic, resulting in far fewer animals needing to be vaccinated (Herd et al., 1999; Chi et al.,

1990).

Pioneering studies using antigens prepared from oncospheres, protoscoleces and membranes that of hydatid cyst induced significant resistance in dogs (Gasser et al., 1988; Heath et al.,1996; Torgerson, 2006; Farhodi et al.,2010). Dogs injected by Freeze-dried protoscoleces could suppress the growth of the terminal segments of worms in a challenge infection (Gemmell, 2001). Oral immunization of dogs with irradiated protoscoleces induces resistance through limitation of the number of established worms that were able to develop to the gravid stage after challenge infection (Budke et al., 2005). Dogs vaccination by excretory-secretory antigens of adult *E. granulosus* worms reduces the worm numbers and causes a highly significant suppression of egg production (Herd et al., 1999; Zhang and Mc Manus, 2008). This study is aimed to determine the immunogenicity of hydatid cyst fluid and protoscoleces under 30 kDa proteins provide protection in experimentally infected dogs with *E. granulosus*.

Materials and Methods

Preparation of the samples: Sheep livers infected with the hydatid cyst were obtained from industrial slaughterhouses around the Tehran Province. The fluid and protoscoleces were aspirated and centrifuged at 4 o c, 2000 × g for 5 minutes and the supernatant was separated. The protoscoleces were washed for 5minutes by PBS (pH 7.2).

Purification of the less than 30 kDa hydatid cyst fluid proteins

After separation of hydatid cyst fluid from liver cyst, 15ml of fluid was poured into a millipore filter (Amicon ultra, Ireland) and centrifuged at 4 o c, 5000 × g for 10 minutes. The filtered fluid proteins were lyophilized and measured were by the Bradford method (Bradford, 1976).

Purification of proteins: Protoscoleces were washed five times with PBS-PMSF (pH, 7.2 and 5mM) and sonicated on ice in a 30HZT ultrasonic disintegrator in ten second bursts with five second intervals. The sonicated materials left at 4?c and then centrifuged at 14,500× g for 30min.The supernatant was poured into a Millipore filter. The filtered fluid was lyophilized and followed by protein concentration measurement by the Bradford method (Bradford, 1976). SDS-PAGE was performed, according to the Laemmli protocol (1970), to determine the prepared proteins.

The Challenged dogs: This work was performed as a cross- sectional study on six dogs (5-6 months old). Dogs were vaccinated against distemper, adenoviruces, parvoviruses and leptospirosis and treated with anti-parasite drugs (combination product: Drontal, Praziquantel 5mg + Pyrantel 10 mg) to eliminate any intestinal helminthes infections. Dogs were labeled in three groups. The first group was injected intramuscularly by 1ml of 30 kDa proteins of hydatid cyst fluid protein (50µg/ml), and the second goup injected intramuscularly 1ml protein (50µg/ml) of protoscoleces, along with 1ml complete freund's adjuvant. The third group, as control, was injected by 1ml deionizer distilled water (placebo).

The second injection was done by using 1 ml of under 30 kDa proteins (50µg/ml) along with 1 ml incomplete freund's adjuvant and the control group was injected by distilled water. Two weeks after the last injection, the dogs, in three groups, were administered with approximately 8000 protoscoleces, with 80% viability.

Blood sampling was done three times, including 2, 4 and 5 weeks post infection, with protoscoleces. The blood was clotted at room temperature for 30 minutes and then at 4^{o}c for 4 hours. The clot was separated from the serum by centrifugation at 3,000× g for 10 minutes and the serum stored in 1ml aliquots at -20^{o}c, until use.

On day 35, after challenged, each dog was killed by intravenous injection of an overdose of Sodium Pentobarbital. Necropsy was performed and the intestines were incubated for 1.5 hours at 37^{o}c. Finally, the contents of intestines were washed and total worms were counted. From each group 20 of worms were stained by acetocarmen method and total worm length, segmentation and maturation were determined. Data was statistically analyzed using SPSS18 software. The ANOVA test was used to

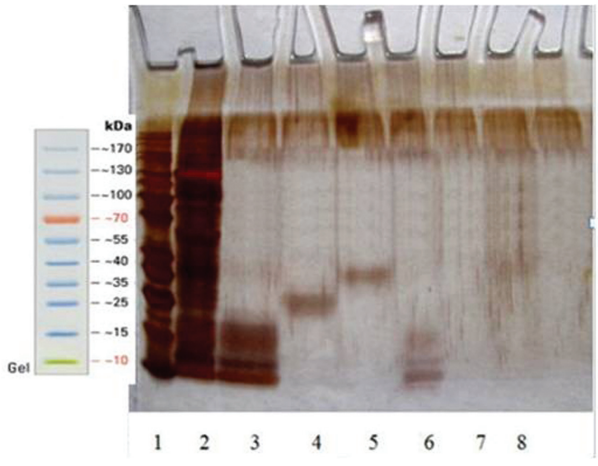

Figure 1: The fractions of under 30KDa proteins using SDS-PAGE with Agno3 staining. 1: Protein ladder, 2: Native hydatid cyst fluid, 3: Lyophilized protein 10-20 KDa, 4: Lyophilized protein 24KDa, 5: Lyophilized protein 30KDa, 6: Unlyophilized protein 10-20 KDa, 7: Unlyophilized protein 24 KDa, 8: Unlyophilized protein 30 KDa.

compare the percentage of worms obtained in the experimental and control groups.

Detection of serum antibody: The ELISA method was used for detection of serum antibody. Preparation of hydatid fluid and protoscoleces antigen was performed based on Rafiei and Craig (2002). The HRP- ELISA plate was read out 450nm on an ELISA reader (Statfax instrument). Cut off values for ELISA were determined according to the Optical Density (OD) of a serum sample from 2 healthy dogs (puppies), two times.

Results

The patterns of Prepared Antigen were assessed by SDS-PAGE to confirm the filtered and lyophilized antigen contents just under 30KDa proteins (Fig1).

Average number of worms recovered in Group I was 197; in Group II was 207; and, Group III was 382. According to the growth rate of adult worms in different groups (Table1) the total length and the length of terminal segment in the control group was significantly more than other groups ($p<0.001$) (Table1).

The levels of antibody in the three groups are shown in Table 2. Antibody levels increased after Ag injection and challenged, with protoscoleces in all groups. The antibody ELISA results did not show significant differences between control and treatment groups (Table2).

Discussion

Control of cystic echinococcosis is one of the most important issues in an endemic area, although not the only one. The potential use of a vaccine in dogs, as a definitive host, is more effective because could reduce the biomass of *E. granulosus* (Zhang and McManus, 2008). Determining the rate of infection and mean abundance in dogs is probably the best index of the degree of transmission of *E.granulosus* in a local region (Benito et al., 2006; Jenkins et al., 2000; Herd et al., 1999). That is essential for the establishment of baseline data on prevalence, and in surveillance of hydatid control programs in endemic areas (Eckert et al., 2001; Eckert et al., 2004). In this study, we used under 30 kDa MW proteins of the hydatid cyst fluid and protoscoleces antigens against *E. granulosus* infection in dogs to evaluate the developmental characteristics. The number of worms in the group which received 30 kDa fluid antigens (Group I) was less than the group which received under 30 kDa protoscolex antigens, and the control group. It has also been shown that the total and terminal segment length of worms in Group I, was smaller than other groups and the size of adult worms in the control group was significantly greater than other groups. This may be due to the effect of under 30 kDa MW proteins, mainly the hydatid cyst fluid antigens.

For unknown reasons a small proportion (3.3%) of protoscoleces readily infected dogs. Although, no significant difference was observed in the antibody level in the three examined groups, the number of worms in groups who received under 30 kDa fluid Ags was less than that group who received under 30 kDa protoscoleces Ags and the control group. Vaccination with soluble protoscoleces antigens and recombinant proteins can induce either total inhibition of egg production or cause delayed embryogenesis. This might be sufficient to reduce

Table 1: Developmental characteristics of *E.granulosus* derived from dogs in three groups at 35 days post infection.

	Total worm length (TWL) (mm)	Length of terminal segment (LTS) (mm)	LTS to TWL ratio (mm) Mean ± SD (Min - Max)
Fluid antigen (groupI)	2.37 ± 0.02	0.9 ± 0.03	0.38 ± 0.02
	(2.35 - 2.42)	(0.89 - 0.93)	(0.38 - 0.41)
Protoscoleces antigen(groupII)	2.72 ± 0.06	1.24 ± 0.06	0.45 ± 0.02
	(2.63 - 2.86)	(1.15 - 1.31)	(0.42 - 0.49)
Control (groupIII)	3.15 ± 0.03	1.63 ± 0.06	0.51 - 0.01
	(3.05 - 3.27)	(1.56 - 1.7)	(0.5 - 0.53)

Table 2: Observance density on 450 nm of serum antibody ELISA in three groups at 35 days post infection.

Groups	Before treatment	Second week Mean±2SD	Forth week Mean±2SD	Fifth week Mean±2SD
I	0.66±0.06	0.792± 0.2	0.953± 0.4	0.914± 0.1
II	0.57±0.1	0.850± 0.06	0.976± 0.78	0.718± 0.4
III	0.61±0.05	0.692± 0.5	0.817± 0.89	0.786± 0.4
Cut off value was 0.7 ± 0.05.				

transmission of infection in endemic areas (Zhang et al., 2003; Zhang et al., 2006).

In previous immunization studies, which relied on the use of somatic antigens, various manifestations of host resistance included high reduction of the worm numbers, worm size, retarded sexual development and egg production suppression (Lightowlers, and Heath, 2004 ; Zhang et al., 2006). Our results showed that less than 30 kDa proteins of hydatid cyst fluid and protoscoleces can be used as a new protective protein for immunization of dogs against echinococcosis.

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