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Detection of *Echinococcus granulosus* infection in dogs using coproantigen enzyme-linked immunosorbent assay

Dalimi[*1], A., Motamedi[2], Gh.R., Syavashi[3], M., Paykari[2], H., Hablolvarid[4], M.H.

1. Department of Parasitology, Faculty of Medical Science, University of Tarbiat Modares, Tehran, Iran
2. Department of Parasitology, Razi Vaccine & Serum Research Institute, Karaj, Iran
3. *Department of Parasitology, Pasteur Institute. Tehran, Iran*
4. Department of Pathology, Razi Vaccine & Serum Research Institute, Karaj, Iran

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ABSTRACT

Cystic Echinococcosis is an important zoonosis in the sheep rising areas of Iran. To develop a simple and reliable diagnostic method for *Echinococcus* infection in definitive hosts, *E. granulosus* polyoclonal antibodies (PolyAbs) were prepared from adult worm in rabbit. A selected PoAb was used for coproantigen detection in faecal samples obtained from animals naturally infected with *Echinococcus* and compared with necropsy method. Finally, the sensitivity and specificity of the test were evaluated. The results indicated *E. granulosus* worms was detected in 36 (43%) of small intestine contents of dogs. The results obtained by CpAg- ELISA test showed 30 (36.14%) positive and 53 (63.86%) negative cases. The sensitivity and specificity of CpAg-ELISA test were evaluated 83.33% and 100% respectively. In conclusion, the present result suggests that, CpAg-ELISA is a valid test for detection of *E. granulosus* infection in living dogs. Thus it is appropriate to apply for epidemiological study.

Keywords: *E. granulosus*, dogs, coproantigen, enzyme-linked immunosorbent assay

INTRODUCTION

Cystic echinococcosis is one of the most important zoonotic helminthiasis in the world caused by the tapeworm *Echinoccocus granulosus*. This infection is also prevalent in sheep raising areas of Iran (Dalimi *et al* 2002). *Echinococcus granulosus* is transmitted between domestic dogs which harbor the adult tapeworm and herbivorous, which act as intermediate host for the cystic larval stage. Infected dogs as the definitive hosts are able to excrete a great number of segments full of eggs with their faeces contaminating the soil and spreading the infection (Benito *et al* 2006). Human infection may occur through direct contact with dogs or environment contamination following ingestion of eggs passed in the faeces of dogs (Benito *et al* 2006, Benito & Carmena 2005). Due to the potential ability of dogs to contaminate the environment, detection of infection in the definitive host has a great importance for epidemiological and ecological studies and also designing an effective strategy for control programs (Dalimi *et al* 2002,

[*]Author for correspondence.Email:dalimi_a@modares.ac.ir

Eckert *et al* 1981). The eggs of all genus belong to taeniidae are identical, so, the differentiation of eggs of taeniidae in faeces of dogs is impossible. For many years the traditional method; intestinal scraping technique and observing the adult worms intestinal content were the only methods for the diagnosis of infection in definitive hosts. Meanwhile, arecoline purgation was the method of choice for the diagnosis of echinococcosis in living dogs. These methods are laborious, expensive procedure, with biological risqué (Schantz *et al* 1995). In recent years the sensitive tests like coproantigen enzyme-linked immunosorbent assay (CpAg-ELISA) were developed by some investigators which is confirmed as the standard protocol by WHO for the diagnosis of infection in dogs (Allan *et al* 1992, Jenkins *et al* 1990). To develop a simple and reliable diagnostic method for *Echinococcus* infection in definitive hosts, *E. granulosus* polyoclonal antibodies (PoAbs) were prepared from adult worm and its sensitivity and specificity of the test were evaluated.

MATERIALS AND METHODS

Inducing the infection in dogs. Fertile sheep hydatid cysts were collected from Tehran abattoir and transferred to parasitology lab of Razi Institute to check the viability of protoscoleses. Then two dogs were infected orally by fertile hydatid cysts. The dogs were kept in separate cages with complete sanitary conditions for 3 months.

Collecting Adult worms. After emerging the parasite eggs in faeces of dogs, the dogs were sacrificed and their small intestines were cut and tied off at both ends and transferred to a beaker containing tap water to parasitology lab. The dog intestines were opened and the worms left overnight to relax and detach from the gut wall. The gut contents were examined in a black-back tray using stereo microscope and the worms were separated according to their morphological characteristics.

Antigen preparation. Somatic antigens were prepared from non-gravid segments of adult *E. granulosus* worms. The worms were washed in PBS and then non-gravid segments were separated and homogenized by freeze-thaw procedure in PBS. The provided suspension was left for few minutes at room temperature. Then the suspension was sonicated at 20 pulse /min by 120% power. The suspension was centrifuged for 30 min at 1000g, at 4 ?C and its supernatant was stored at -70?c until used. The antigen concentration for using in ELISA test was optimized at 50 mg/ml using Lowery method.

Polyclonal antibody preparation. A white rabbit was immunized 3 times by subcutaneous injection of 300 µg of antigen. The first injection contained the somatic *E. granulosus* antigen plus complete Ferund's Adjuvant. Two weeks later first booster, contained the same antigen plus Incomplete Ferund's Adjuvant was injected. The third injection was carried out with the antigen in normal saline. Seven days after the last injection the whole blood of the hyper immunized rabbits were collected.

Faecal samples. During three years, 83 (63 male and 20 female) dead stray dogs which found killed by car accidents in roads were collected. Faecal samples were collected directly from the dog intestines and mixed with PBS at the ratio of 1:4(v/v) containing 0.04% NaN3, 0.05% Bovine hemoglobin (Fluka**,** Backs**,** Switzerland) and 0.3% Tween-20. For safety purposes, faecal samples were frozen at -80?C for at least one week and then stored at -20?C until use. For testing by ELISA, the samples were centrifuged at 3000g for 10 min at room temperature and the supernatants were collected.

Coproantigen enzyme-linked immunosorbent assay. CA-ELISA was performed with some modifications according to Allan et al 1992. In brief, about 5µg/ml of hyperimmune rabbit

immunoglobulin against *E. granulosus* somatic antigens diluted in carbonate-bicarbonate buffer was used to coat (100µl per well) plates (Immunolin, Dynalech) overnight at 4 °C. Plates were washed 3 times by PBS 0.1% tween 20 and blocked with 100 µl per well of PBS-0.3% TW20 for 1hr at room temperature. Faecal supernatant samples were assayed at 1:2 dilution in PBS and incubated twice for 1hr at 37^{0}C, then washed 3 times in PBS-0.1% TW20. One hundred microlitres per well of peroxidase –conjugated rabbit immunoglobuline anti-*Echinococcus granulosus* somatic products (CheKit Echinotest Bommeli Diagnostic, Switzerland) diluted 1:200 was incubated for 1hr at room temperature and plates subsequently washed as above. The substrate solution was 5amino salicylic acid (Sigma) in 0.1 M phosphate buffer PH=6 containing 1mM of EDTA and 0.005% hydrogen peroxide. The measurement was performed at the wavelength of 450 nm after 25 minutes by micro plate reader (Dynatech MR 500). The cut -off absorbance value was based on the mean absorbance of the faecal supernatants from helminth dogs (previously diagnosed by post–mortem intestinal examination) plus 3SD(Mean Absorbance ±3SD). Diagnostic performance of CpAg-ELISA was evaluated using necropsy as gold standard. Antigen concentration for ELISA was optimized by checkerboard titration.

RESULTS

Necropsy examination. *E. granulosus* worms were detected in 36 (43%) of small intestine contents of dogs. The worm burden for *E. granulosus* was from 33 to 4322 adult worms. Several cestodes other than *E. granulosus* including *Taenia hydathigena*, *Taenia ovis* and *Taenia pisiformis* were also found.

CpAg- ELISA test. The results obtained by CpAg- ELISA test showed 30 (36.14%) positive and 53 (63.86%) negative cases. The sensitivity and specificity of CpAg-ELISA test are evaluated 83.33% and 100% respectively (Tables 1 & 2). In addition, positive and negative predictive values for this test are calculated as 100% and 88.68% respectively (Table 2).

Table 1. Comparison of coproantigen (CpAg) ELISA assays and necropsy for the detection of *Echinococcus granulosus* in the studied dog population (n=83)

	Necropsy results		Total (=83)
	Positive (n=36)	Negative (n=47)	
CpAg ELISA (+)	30 (83.33%)	0 (0%)	30 (36.14%)
CpAg ELISA (–)	6 (16.66%)	47 (100%)	53 (63.86%)

Necropsy was considered as the gold standard method (Benito & Carmens, 2005).

Table 2. Validity of coproantigen (CpAg) ELISA assays for the detection of *Echinococcus granulosus* in the studied dog population (Necropsy was considered as the gold standard)

Tests	%
Sensitivity	83.33
Specificity	100
Positive predictive value	100
Negative predictive value	88.68
False positive rate (α)	0
False negative rate (β)	16.67
Likelihood-ratio positive	0
Likelihood-ratio negative	0.1667

DISCUSSION

For detecting *E.granulosus* in living definitive host, different methods have been developed. In stool examination of dogs, eggs from different taeniid cestodes cannot be differentiated by light

microscopy, in addition, egg production may be irregular, so the test has low sensitivity and specificity. Although, examination of the small intestine at necropsy of dogs was the gold standard method for the detection of *E. granulosus* (Benito & Carmens 2005), but this method is dangerous, laborious and ethically questionable procedure so, is not suitable for mass screening. Meanwhile, arecoline purgation has been evaluated the method of choice for the diagnosis of echinococcosis in living dogs. But it is also a laborious, expensive procedure, with biological risque and low sensitive (65%) (Schantz *et al* 1995). Similarly, serological screening generally is considered to be not suitable for the diagnosis of infections with *Echinoccocus spp*, because of poor correlation between antibody titers and the presence of the worm (Deplazes *et al* 1992). In the last decade, some specific methods such as detection of specific coproantigens and PCR procedure on DNA originating eggs isolated from faecal samples have been developed and evaluated by some investigators (Fraser & Graig 1997). Detection of the parasite antigens in faecal samples of dogs (coproantigens) by ELISA using antibodies against adult somatic antigens (Allan *et al* 1992), and excretory–secretory products from proglottids (Deplazes *et al* 1992) or protoscoleces (Benito & Carmena 2005) showed a higher diagnostic sensitivity than Ab ELISA (Craig *et al* 1995, Benito *et al* 2006). In fact, ELISA has showed variable sensitivities, ranging from 40 to 90% (Benito *et al* 2006, Gasser *et al* 1994, Jenkins *et al* 1990). In the present study, CpAg- ELISA test was found to be highly sensitive and specific in the detection of *Echinococcus granulosus* coproantigens. The sensitivity and specificity of the test was evaluated 83.33% and 100% in our study however, it was reported 88 and 95% by Lopera et al (2003), and also 98.1 and 100% by Buishi et al (2005), respectively. High degrees of specificity were reported by Allan et al (1992), Malgor et al (1997) and Prathiush et al (2008). Cross-reactivity with *Taenia* spp. was observed in some studies (Christofi *et al* 2002, Lopera *et al* 2003, Casaravilla *et al* 2005, Prathiush *et al* 2008). In our study although several cestodes like *Taenia hydathigena, Taenia ovis and Taenia pisiformis* were also found in small intestine of free-*E. grunlosus* dogs but no cross reactivity was found. Like Benito et al (2006) CpAg ELISA assays showed a very high negative predictive value, a characteristic that make them specially suited for the mass-screening of dog populations with low prevalence of *E. granulosus*. But, positive predictive values of CpAg ELISA techniques in Benito et al. (2006) study was relatively low which is in contrast with our results (Benito et al 2006). Parasite burden may effect on validity of the test. Cohen et al. (1998) reported high specificity (>95%) and sensitivity (100%) in sandwich ELISA with worm counts greater than 20 and ELISA results correlate well with the worm burden in the dog intestine (Craig *et al* 1995). In our result the worms were counted from 33 to 4322, which is highly correlated with obtained test validity. Considering the reliable results obtain from many studies, nowadays coproantigen tests have applied successfully in epidemiological studies in many countries including Spain (Deplazes *et al* 1994), Finland (Hirvel?-Koski *et al* 2003), Iran (Siavashi & Motamedi 2006, Zare-Bidaki *et al* 2009), Swiss (Deplazes *et al* 1992, Deplazes *et al* 1999), France (Magnaval *et al* 2004), Slovakia (Reiterova *et al* 2005), Poland (Machnicka *et al* 2003), Norway (Fuglei *et al* 2008), Japan (Kohno, 1991, Sakai 1996, Sakai *et al* 1998, Morishima *et al* 1999, Kamiya *et al* 2007), Australia (Jenkins *et al* 2000), Romania (Stefania *et al* 2006) as well as for surveillance of canine echinococcosis in a hydatid control programme in Cyprus (Christofi *et al* 2002). In conclusion, the present result suggests that, CpAg-ELISA is a valid test for detection of *E. granulosus* infection in living dogs. Thus it is appropriate to apply for epidemiological studies.

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