Evaluation of *Echinococcus granulosus* coproantigens by Dot-blotting in dogs

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Summary

Definitive hosts of the *Echinococcus granulosus* (*E. granulosus*) parasite are carnivores such as dogs, wolves and foxes. Detection of this parasite through faecal examination is not possible. In this study, dot-blotting test for *E. granulosus*-specific coproantigens has been evaluated in dog. Three 2–3-month-old puppies were treated with piperazine and then faecal samples were collected as pre-infection samples. Seven days later, hydatid cysts from livers and lungs of sheep were fed to the puppies. Faecal samples were collected weekly for five weeks as post-infection samples. Soluble protein of pre- and post-infection faecal samples were prepared and dot-blotting test was conducted. In parallel experiments, the presence of *E. granulosus* eggs and also dot-blotting test were evaluated in 15 faecal samples of dogs collected from Razi Veterinary Hospital in Mashhad. For the detection of protein bands in pre-infection and fifth-week post-infection samples, polypeptide profile was analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The results showed that incremental spot colours was observed in samples of experimentally infected dogs collected from the first to fifth post-infection weeks. In dot-blot analysis of faecal samples in 15 dogs, 4 samples were positive, and also these four samples were positive for *E. granulosus* eggs. In SDS-PAGE, one band in pre-infection and four bands in fifth-week post-infection samples were observed. The molecular weight of pre-infection sample of experimentally infected dogs was 16 kDa and the molecular weights of the samples collected five weeks post-infection were 14, 22, 36 and 45 kDa, respectively. In conclusion, the results of this experiment showed that the dot-blotting method does not produce a reliable outcome. For evaluation of the specific coproantigens of *E. granulosus* in dogs, coproantigen-ELISA test is needed.

Key words: *Echinococcus granulosus*, Coproantigens, Dot-blotting, Dog

Introduction

Over the last few years, immunodiagnostic techniques based on the identification of parasitic antigens in recently emitted faecal material, or faecal extracted directly from the rectum of the dog (Allan *et al*., 1992; Craig, 1997) have developed as alternatives for the surveillance of canine echinococcosis. The immunological methods such as ELISA and dot-blotting are highly accurate and reliable techniques for serodiagnostic purposes, although their accuracy is largely dependent on the quality of the antigenic source used (Carmena *et al*., 2007). Several groups have developed ELISA for detecting *Echinococcus granulosus* coproantigen in dog populations in various endemic areas (Zariffard *et al*., 1999; El-Shehabi *et al*., 2000; Cavagión *et al*., 2005). Comparison between coprodiagnosis and serological methods revealed that detection of coproantigens was 2.5 times more sensitive than antibody detection (Craig *et al*., 1995). ELISA is a method with high accuracy in the detection of infected dogs with *E. granulosus* on the first post-infection week but this technique is costly, laborious and cumbersome for large-scale epidemiological studies (Ahmad and Nizami, 1998). Dot-blotting is an ELISA-based method that

detects the *E. granulosus*-specific coproantigens in the faecal samples. Detection of parasite-specific coproantigens in the faeces of infected hosts is used for the diagnosis of variety of intestinal protozoan infections (Baumann and Gottstein, 1987). Dot-blotting has also been used for the detection of intermediate hosts such as sheep and human and the results were satisfactory. Electrophoresis was also conducted for the detection of parasite protein bands. A system of canine echinococcosis surveillance based on the use of the diagnostic complex, copro-ELISA plus copro-Western blot using dry faeces collected from the environment, without identification of the emitting dog and without the application of taeniafuges, has been recently proposed (Guarnera *et al.*, 2000). In *E. granulosus* carrier dogs, the sensitivity and specificity of this system is 100%. Copro-ELISA test has been used for control programs in Cyprus, Spain and Peru (Deplazes *et al.*, 1994; Christofi *et al.*, 2002; Lopera *et al.*, 2003) and data were expressed in surveillance systems as percentages of infected dogs.

The aim of the present study was to detect *E. granulosus*-specific coproantigen in the faecal samples of the infected dogs by dot-blotting method and to determine the polypeptide profile of faecal samples of *E. granulosus* (pre-infection and fifth-week post-infection) by SDS-PAGE.

Materials and Methods

Faecal samples

Three 2–3-month-old puppies were treated with piperazine oral solution. Faecal samples from puppies were collected 24 h after using piperazine as pre-infection samples. At the beginning of the second week, hydatid cysts from lungs and livers of sheep taken from Mashhad slaughterhouse were fed to the puppies. At the end of every week, faecal samples were collected for five weeks. The samples were transferred into freezer. Then the faecal samples were thawed and mixed with 0.05 M bicarbonate buffer at a ratio of 1:10 and shaken. The samples were centrifuged at 2000 g for 30 min and the supernatants stored at 4ºC as a source of antigen. The protein concentration of the samples was determined by the method of Bradford (1976).

Collecting sera

One dog was treated with piperazine and then fed with cystic livers and lungs. After three months, the dog was euthanized and mature parasites (*E. granulosus*) were removed from the small intestine. The sample was freeze-thawed three times and mixed with four volumes of PBS, pH = 7.4, containing sodium azide at 0.1 mg/ml. After that, the sample was sonicated in a 200 W ultrasonic disintegrator (Hielscher, Germany) on ice for three min and then homogenized. The preparation was left on ice for one h and centrifuged for 30 min at 10,000 g and then filtered (0.22 µm) (Ahmad *et al.*, 2001). Protein concentration of *E. granulosus* was determined by the method of Bradford (1976) and used as antigen source. A mixture of 1 mg of *E. granulosus* protein dissolved in 1 ml of PBS plus 1 ml of Freund's complete adjuvant (FCA) was prepared and two ml of this antigen was inoculated three times in a rabbit subcutaneously. After two weeks, the rabbit was re-injected with the same preparation, except that FCA was replaced by Freund's incomplete adjuvant (FIA). The third injection was conducted without any adjuvant on day 28. Two weeks after the third injection, blood sample was taken from the rabbit and serum was separated as the antibody source and stored at -20ºC until used.

Dot-blotting

The faecal antigens of three puppies were spotted on the nitrocellulose sheets and allowed to dry. The test strips were washed with 10 mM of PBS, PH = 7.4, for 3 × 10 min. Non-specific binding sites on strips were blocked with PBS plus 2% BSA for one h at room temperature (RT) on a rocker platform. The membrane strips were washed with 10 mM of PBS, PH = 7.4, for 3 × 10 min and incubated for three h in serum at a dilution of 1:10. Then the membranes were washed in 10 mM of PBS containing 0.05% Tween 20 for 3 × 10 min. The strips were incubated with the relevant alkaline phosphatase conjugates with appropriate

conjugated sheep anti-rabbit IgG (Sigma), followed by incubation with PBS (1/1000) plus 1% BSA for three h at RT on a rocker platform. The strips were washed as mentioned above and the reaction developed using 3,3'-Diaminobenzidine (DAB) or 3, 3',5,5'-Tetramethylbenzidine (TMB) colour. The reaction was stopped by double distilled water and was observed by naked eye. Dot-blotting was conducted in faecal samples of 15 dogs that had been collected from Razi Veterinary Hospital in Mashhad. These samples were studied with microscopic method in order to detect the Taenia eggs.
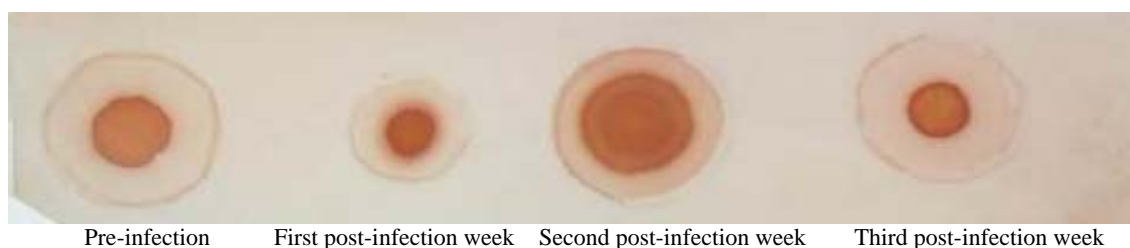
Sodium dodecyl sulphate-polyacryl-amide gel electrophoresis (SDS-PAGE)

For the separation of proteins, discontinuous sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 12.5% concentration under denaturated conditions was carried out as described by Laemmli (1970). The solutions
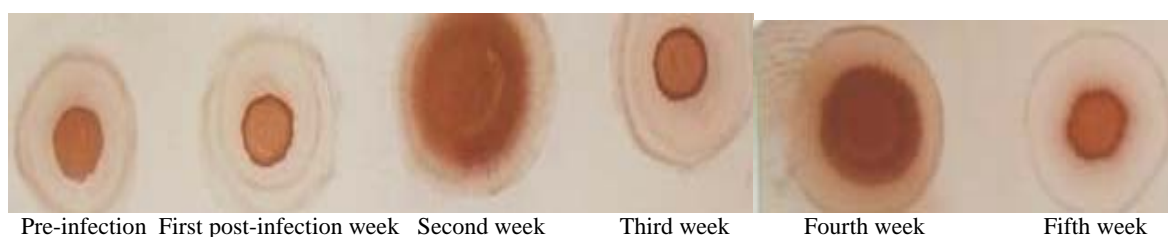
were poured into the mould as a separating gel (Biogene). The gel was allowed to set and then the stacking gel was poured on top of the running gel with the comb in place. The gel was run in a Biogene electrophoresis apparatus for 4 h in a gel running buffer. The gel was stained overnight in Coomassie blue followed by soaking in destaining solution with multiple changes for 4 h on gentle rocking platform. The gel was stored at 4°C in a solution containing 30% v/v methanol and 10% v/v glycerol. Wide molecular weight (Sigma) was used for determination of molecular weights.
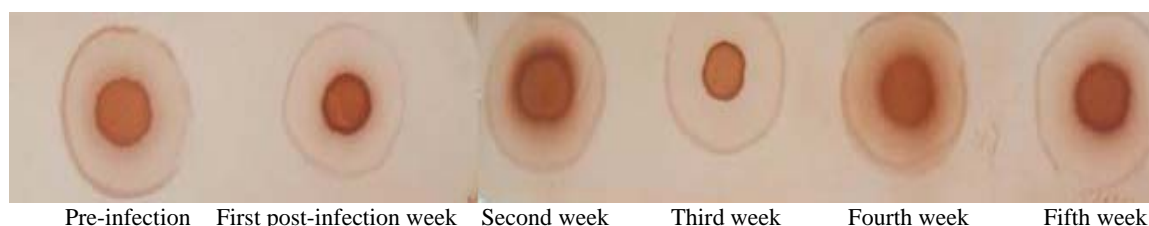
Results

Dot-blot analysis of the faecal extracts collected from the first to fifth-week post-infection in three experimentally infected dogs revealed colour increments on the strips (Fig. 1). The results showed that four out of 15 samples were positive for the existence of Taenia eggs and also dot-blot

| Pre-infection | First post-infection week | Second post-infection week | Third post-infection week |

Puppy 1

| Pre-infection | First post-infection week | Second week | Third week | Fourth week | Fifth week |

Puppy 2

| Pre-infection | First post-infection week | Second week | Third week | Fourth week | Fifth week |

Puppy 3

Fig. 1: Results of dot-blotting by DAB solution in experimentally infected puppies in pre- and post-infection weeks (puppy 1 died at the end of the third post-infection week)

analysis of these 4 samples proved to be positive (Fig. 2). Absorbance values in the samples of experimental puppies are shown in Table 1. The molecular weight of pre-infection sample was 16 kDa and the molecular weight of samples collected five weeks post-infection were 14, 22, 36 and 45 kDa, respectively (Fig. 3).

Discussion

The most important advantage of coproantigen ELISA over conventional serum antibody assays is that it shows current infection (Allan *et al*., 1992; Deplazes *et al*., 1999; Jenkins *et al*., 2000). In a given population of dogs exposed to *E.*

granulosus, the sensitivity of coproantigen ELISA was superior to the use of antibody detection (Craig *et al*., 1995). Coproantigens are detectable during patent period as well as pre-patent period, and disappear within 2-5 days after the elimination of *Echinococcus* worms. Coproantigens remain stable for at least five days at room temperature. They can be fixed in 5-10% formalin and then be stored for weeks at 4ºC or at room temperature (Jenkins *et al*., 2000; Craig *et al*., 2003).

The sensitivity and specificity of the coproantigen ELISA test for dogs was 88 and 95% by Lopera *et al*. (2003), and also 98.1 and 100% by Buishi *et al*. (2005), respectively. Coproantigen tests for *E. granulosus* have now been applied
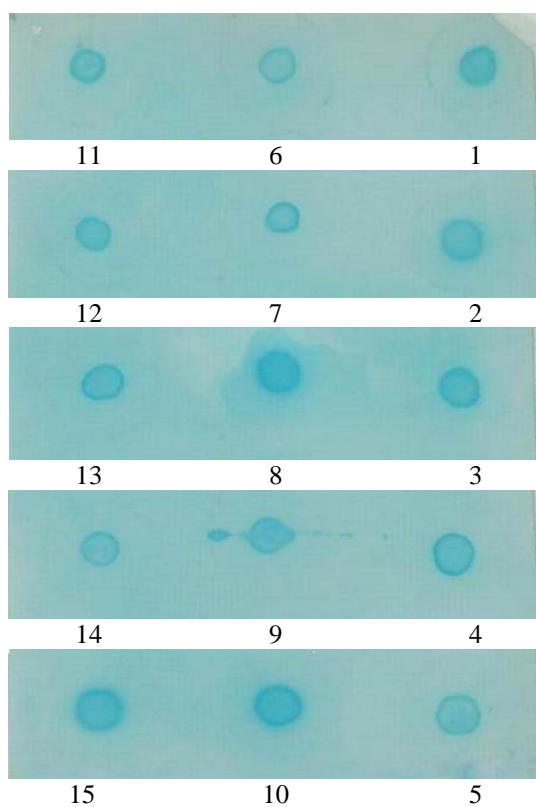
Fig. 2: Results of dot-blotting by TMB solution in 15 faecal samples collected from Mashhad Veterinary Hospital (Taenia eggs were observed in samples 1, 7, 8 and 9)
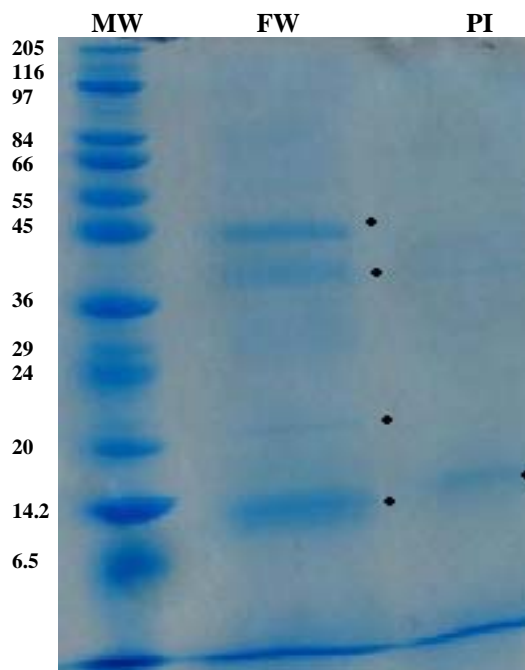
Fig. 3: Polypeptide profile of faecal samples analysed by SDS-PAGE. In the pre-infection week only one band (16 kDa) and in the fifth post-infection week four bands (14, 22, 36 and 45 kDa) were observed. MW: standard molecular weight, FW: fifth-week and PI: pre-infection week

Table 1: Absorbance value results in puppy samples (puppy 1 died at the end of the third post-infection week)

Absorbance	Weeks					
	Pre-infection	First post-infection	Second post-infection	Third post-infection	Fourth post-infection	Fifth post-infection
Puppy1	336	356	312	125	-	-
Puppy 2	250	171	469	102	343	201
Puppy 3	108	207	465	130	366	232

successfully in epidemiological studies in owned dog populations in countries such as Spain (Deplazes *et al*., 1994), Finland (Hirvelä-Koski *et al*., 2003) and for surveillance of canine echinococcosis in a hydatid control programme in Cyprus (Christofi *et al*., 2002). Because of simplicity of dot-blotting technique, in the present study, this technique has been evaluated for *E. granulosus*-specific coproantigens in dogs.

In the present study, incremental spot colours was observed in samples of experimentally infected dogs collected from the first to fifth post-infection weeks. In another experiment, four bands in the fifth-week post-infection sample were observed by SDS-PAGE and a band was also observed in pre-infection sample. Ahmad and Nizami (1998) reported that in fourth-week post-infection, a prominent reaction was observed as revealed by the intensity of the spot. However, in their study, no increment was observed in spot colours and all samples from different weeks were similar. They determined four bands in SDS-PAGE being absolutely sharp in the fourth-week post-infection samples, but the molecular weight of bands were not mentioned. In electrophoresis on successive pre- and post-infection samples, it was specified that the four bands grew gradually sharper. The results showed that these bands did not exist in pre-infection samples. Bands were gradually appeared and were probably related to *E. granulosus*.

It is concluded by Ahmad and Nizami (1998) that faecal antigens can be detected by ELISA at early stages of infection. Elayoubi *et al*. (2003) showed that panels of positive and negative faecal coproantigen supernatants were subjected to SDS-PAGE under reducing or non-reducing conditions and also detectable by silver staining. The profiles of both negative and positive faecal samples were similar and showed a complex pattern of polypeptide bands ranging from 12 to 250 kDa. However, no specific differences were detected in profiles between faecal samples of positive or negative coproantigen status by Western blot. The use of a lower concentration of SDS (8%) in the gels or gradient gels (4-20%) did not improve profiles for high

molecular mass molecules. It has been suggested that coproantigens detected in faeces of *E. granulosus*-infected dogs are large molecular weight molecules (Elayoubi and Craig, 2004).

Although the copro-dot-blot system has been promising for its application in epidemiological surveillance systems to detect echinococcosis in dog, the value of this method is doubtful and it is necessary to evaluate the *E. granulosus*-specific coproantigens in dog by coproantigen-ELISA.

Acknowledgements

References

Ahmad, G and Nizami, WA (1998). Coproantigens: early detection and suitability of an immunodiagnostic method for echinococcosis in dogs. Vet. Parasitol., 77: 237-244.

Ahmad, G; Nizami, WA and Saifullah, MK (2001). Analysis of potential antigens of protoscoleces isolated from pulmonary and hepatic hydatid cysts of *bubalus bubalis*. Comp. Immunol. Microbiol. Infect. Dis., 24: 91-101.

Allan, JC; Craig, PS; Garcia Novel, J; Mencos, F; Liu, D; Wang, Y; Wen, H; Zhou, P; Stringer, R; Rogan, M and Zeyhle, E (1992). Coproantigen detection for immunodiagnosis of echinococcosis and taeniasis in dogs and humans. Parasitology. 104: 347-356.

Baumann, D and Gottstein, B (1987). A double-antibody sandwich ELISA for the detection of *Entamoeba histolytica* antigen in stool samples of humans. Trop. Med. Parasitol., 38: 81-85.

Bradford, MM (1976). A rapid and sensitive method for quantitation of microorganisms quantities of protein utilizing the principle of protein dye binding. Ann. Biochem., 72: 248-254.

Buishi, IE; Njoroge, EM; Bouamra, O and Craig, PS (2005). Canine echinococcosis in northwest Libya: assessment of coproantigen ELISA, and a survey of infection with analysis of risk-factors. Vet. Parasitol., 130: 223-232.

Carmena, D; Benito, A and Eraso, E (2007). Recent advances in the immunodiagnosis of human cystic echinococcosis. Enferm. Infecc. Microbiol. Clin., 25: 263-269.

Cavagión, L; Perez, A; Santillan, G; Zanini, F; Jensen, O; Saldía, L; Diaz, M; Cantoni, G; Herrero, E; Costa, MT; Volpe, M; Araya, D; Rubianes, NA; Aguado, C; Meglia, G; Guarnera, E and Larrieu, E (2005). Diagnosis of cystic echinococcosis on sheep farms in the south of Argentina: areas with a control program. Vet. Parasitol., 128: 73-81.

Christofi, G; Deplazes, P; Christofi, N; Tanner, I; Economides, P and Eckert, J (2002). Screening of dogs for *Echinococcus granulosus* coproantigen in low endemic situation in Cyprus. Vet. Parasitol., 104: 299-306.

Craig, PS (1997). Immunodiagnosis of *Echinococcus granulosus* and a comparison of techniques for diagnosis of canine echinococcosis. In: Andersen, FL; Ouhelli, H and Kachani, M (Eds.), *Compendium on cystic echinococcosis in Africa and Middle Eastern countries with special reference to Morocco.* Provo, UT, Brigham Young University, Print Services. PP: 85-118.

Craig, PS; Gasser, RB; Parada, L; Cabrera, P; Parietti, S; Brgues, C; Acuttis, A; Agulla, J; Snowden, K and Paolillo, E (1995). Diagnosis of canine echinococcosis: comparison of coproantigen and serum antibody test with arecoline purgation in Uruguay. Vet. Parasitol., 56: 293-301.

Craig, PS; Rogan, MT and Campos-ponce, M (2003). Echinococcosis: disease, detection and transmission. Parasitology. 127: S5-S20.

Deplazes, P; Alther, P; Tanner, I; Thompson, RCA and Eckert, J (1999). *Echinococcus multilocularis* coproantigen detection by enzyme-linked immunosorbent assay in fox, dog and cat populations. J. Parasitol., 85: 115-121.

Deplazes, P; Jimenez-Palacios, S; Gottstein, B; Skaggs, J and Eckert, J (1994). Detection of *Echinococcus* coproantigens in stray dogs of northern Spain. Appl. Parasitol., 35: 297-301.

Elayoubi, FA and Craig, PS (2004). *Echinococcus granulosus* coproantigens: chromatographic fractionation and characterization. Parasitology. 128: 455-465.

Elayoubi, FA; Fraser, A; Jenkins, DJ and Craig, PS (2003). Partial characterisation of carbohydrate-rich *Echinococcus granulosus* coproantigens. Int. J. Parasitol., 33: 1553-1559.

El-Shehabi, FS; Kambawi, SA; Schantz, PM; Craig, PS and Abdel-Hafez, SK (2000). Diagnosis of canine echinococcosis: comparisson of coproantigen detection with necropsy in stray dogs and red foxes from northern Jordan. Parasite. 7: 83-90.

Guarnera, E; Santillán, G; Bottinelli, O and Franco, A (2000). Canine echinococcosis: an alternative for surveillance epidemiology. Vet. Parasitol., 88: 131-134.

Hirvelä-Koski, V; Haukisalmi, V; Kilpelä, SS; Nylund, M and Koski, P (2003). *Echinococcus granulosus* in Finland. Vet. Parasitol., 111: 175-192.

Jenkins, DJ; Fraser, A; Bradshaw, H and Craig, PS (2000). Detection of *Echinococcus granulosus* coproantigens in Australian canids with natural or experimental infection. J. Parasitol., 86: 140-145.

Laemmli, UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227: 680-685.

Lopera, L; Moro, P; Chavez, A; Montes, G; Gonzales, A and Gilman, R (2003). Field evaluation of a coproantigen enzyme-linked immunosorbent assay for diagnosis of canine echinococcosis in a rural Andean village in Peru. Vet. Parasitol., 117: 37-42.

Zariffard, MR; Deplazes, P; Basher, N; Motamedi, GR and Nazari, JH (1999). Detection of *Echinococcus granulosus* in carnivores in Iran. Arch. Int. Hidatidol., 23: 264.