Sero-diagnosis of *Echinococcus granulosus* infection in dogs using faecal supernatant antigen

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Abstract In the present study, sero-diagnosis of *Echinococcus granulosus* infection in dogs was studied using 250 field serum samples, collected from stray dogs. The three serological techniques viz., latex agglutination test (LAT), Dot-ELISA and enzyme immunotransfer blot (EITB) were used to detect *E. granulosus* specific antibodies using fecal supernatant antigen. The LAT detected positivity in 53 (21.2 %) serum samples with the sensitivity and specificity of 100 and 78.80 % respectively. Whereas, the Dot-ELISA, showed positivity in 45 (18.0 %) samples and the sensitivity and specificity were 100 and 82.0 % respectively. The specific antibodies were detected in 47 (18.80 %) serum samples by EITB and the polypeptides of 45 and 34 kDa were identified in all the positive serum samples. The sensitivity and specificity of EITB were found to be 100 and 86.80 %, respectively.

Keywords Dog · *E. granulosus* · Serodiagnosis · LAT · Dot-ELISA · EITB

Introduction

Echinococcosis is caused by dwarf dog tapeworm *Echinococcus granulosus* is highly endemic and considered as one of the most important parasitic disease worldwide especially in areas where hygienic conditions and education are poor. The disease is spreading because of lack of appropriate legislation on animal slaughter, dog management and sanitary facilities (Schwabe 1986). The parasite is adapted to definitive hosts of the family canidae and a wide range of intermediate hosts including human beings, domestic, wild herbivores and omnivores (Torgerson and Health 2003).

The definitive host range for the *E. granulosus* is more restricted than the intermediate host range. According to the control perspective, the diagnosis of the adult stages of these parasites is more important than that of the cystic stage (Allan and Craig 2006). The development of sensitive and specific ante-mortem diagnostic methods for the detection of canine echinococcosis is important for epidemiological baseline data and for surveillance of hydatid control programmes. Screening of dogs for *E. granulosus* has traditionally been done by arecoline purgation followed by examination of the purge. Although, the specificity of purgation can be 100 %, it is time-consuming, biohazardous, and has variable sensitivity, in addition to requirement of trained personnel (Eckert et al. 2001).

Clinical diagnosis of echinococcosis has always been problematic because of the variety and non-specificity of symptoms. A rapid and simple diagnostic method is needed in order to conduct epidemiological surveys successfully. For these reasons, immunodiagnostic tests play an important role. The immunological methods vary in their design and adaptability and are useful for the detection and quantification of antigens and antibodies.

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Keeping the above in view, the current study was undertaken to assess the various sero-diagnostic techniques for diagnosis of *E. granulosus* infection in dogs by using fecal supernatant antigen.

Materials and methods

Collection of serum samples

Field sera

A total of 250 blood samples were collected from stray dogs live in and around organized slaughter house without EDTA for serum separation. In the laboratory, the serum samples were separated by centrifugation, aliquoted and stored in the deep freezer at -20°C till further use.

Positive control sera

The hyper immune serum (HIS) was raised in rabbit against fecal supernatant antigen of *E. granulosus*. About 0.5 ml (500 μg) of antigen was mixed with equal volume of Freund's complete adjuvant (FCA) and the mixture was injected subcutaneously to the rabbit. After 7 days, the antigen injection was repeated with Freund's incomplete adjuvant (FIA). Another three injections were given at weekly intervals with the same concentration of antigen. Ten days after the final injection, blood was collected from rabbit and serum separated under sterile condition, aliquoted and stored at -20°C till further use. It was tested by DID (Double Immuno Diffusion) with corresponding antigen for the presence of antibodies and used as true positive serum.

Negative control sera

In the study, 2 weeks old dog serum and uninfected control sera of dogs were used as negative controls.

Preparation of fecal supernatant antigen (F/S Ag)

The fecal supernatant antigen was prepared as per the procedure of Elayoubi and Craig (2004) with slight modification. The fecal sample collected from rectum of two dogs naturally infected with echinococcus worm was used for preparation of F/S antigen. Two grams of fecal sample was taken in a 15 ml centrifuge tube and incubated at 70°C for 12 h. Then the contents were mixed vigorously with 1 % formalin solution containing 0.3 % Tween-20 in 1:2 ratios and centrifuged at 2,000 rpm for 20 min at room temperature. The clear supernatant separated was aliquoted and stored at 4°C till further use.

Protein estimation

The protein concentration of fecal supernatant antigen was estimated as described (Bradford 1976).

Latex agglutination test (LAT)

The stock suspension of 0.81 μm sized polystyrene latex particles (Sigma, Co., USA) was diluted 1:100 with Glycine buffered saline (pH 8.2–8.4). Then 0.5 ml of diluted latex was added to 0.5 ml of the antigen and was shaken for 30 min at room temperature. Bovine serum albumin and Tween-20 were added to a final concentration of 1 and 0.1 %, respectively to minimize or eliminate non-specific agglutination reaction and was stored at 4°C until use.

Fifty micro liter of latex reagent was mixed with an equal volume of serum on a cavity glass slide, rocked gently with orbital motion for 15 min and signs of agglutination were recorded. The results of LAT were determined by visual agglutination reaction, which was scored as 1+, 2+, 3+ and 4+ reactions. The agglutination reaction of 3+ and 4+ were considered positive and 1+ and 2+ as negative. The negative reaction was indicated by uniform turbidity without agglutination.

Dot-ELISA

Dot-ELISA was carried out as per El-Missiry et al. (1990) with slight modifications. In brief, 1 cm^2 of nitrocellulose membrane (0.45 μm porosity) disc was cut and placed in the wells of sterile 96 well polystyrene perplex plates. Then, 2 μl of fecal supernatant antigen containing 1 μg of protein was dot blotted on to the dull side of filter discs and incubated at room temperature for 1 h. To each well containing the discs, 100 μl of blocking buffer was added after washing and incubated at room temperature for 1 h. Further, after washing, 100 μl of diluted serum (1:100) was added to each well in duplicates and incubated for 1 h. Then, 100 μl of HRP conjugated anti-dog IG (1:1,000) diluted in 5 % skim milk powder was added and incubated at 37°C for 1 h. After washing, 100 μl of *ortho*-dianisidine dihydrochloride (ODD) substrate solution was added and shaken for 1 min and incubated at room temperature for color development. The color reaction was stopped by washing the membrane discs in distilled water and read for positive reactions which appeared as clearly defined brown dots.

Enzyme immuno transfer blot (EITB)

The EITB was carried out as per the method of Towbin et al. (1979). SDS-PAGE was carried out first as per description of Laemmli (1970) using discontinuous gel. The fecal supernatant antigen (25 μg) along with the

protein marker of molecular range 14.3–97.4 kDa (Bangalore Genei) was electrophoresed. After electrophoresis, the marker lane was cut and stained with Coomassie brilliant blue R-250 and the remaining gel was used for electro transfer blot. The polypeptides from the gel were transferred on nitrocellulose membrane by trans-blot semidry electrophoresis transfer cell (BioRad) and the membrane was immersed in blocking buffer overnight at 4 °C, then incubated with diluted serum (1:100) washed and incubated in 1:1,000 HRP-conjugated with anti-dog antibody (IG). After washing, 100 µl of *ortho*-dianisidine dihydrochloride (ODD) substrate working solution was added and color reaction was allowed to develop. The results were read by comparing with the standard molecular weight protein marker stained with coomassie brilliant blue R-250 as described earlier.

The sensitivity and specificity of the serological tests was calculated by using

$$\text{Sensitivity: } \frac{\text{True positive}}{\text{True positive} + \text{false negative}} \times 100$$

$$\text{Specificity: } \frac{\text{True negative}}{\text{True negative} + \text{false positive}} \times 100.$$

Statistical analysis

The data were analyzed by Chi square test using the software Graph pad prism.

Results and discussion

The protein concentration of fecal supernatant antigen was estimated to be 1,150 µg per ml of antigen. The specific antibodies of *E. granulosus* were detected by using three serological tests viz., LAT, Dot-ELISA and EITB with fecal supernatant antigen.

The LAT was conducted on 250 serum samples collected from stray dogs, 53 (21.2 %) samples gave positive reaction (Fig. 1) and the sensitivity and specificity was found to be 100 and 78.80 %, respectively (Fig. 2). This is in accordance with the Szyfers and Kagan (1963) used modified latex agglutination test for diagnosis of hydatidosis in human beings and found 100 % sensitivity and 97 % specificity. Similarly, Shimizu (2000) used LAT for the detection of *E. multilocularis* copro-antigens in the definitive host and found 47 and 61 % sensitivity and 94 and 86 % specificity with non-heated and heated faecal samples of wild foxes, respectively.

The higher sensitivity and moderate specificity in the present study could be due to cross reactive nature of antigen with other parasitic antigens. This could possibly due to sharing of common epitopes between closely related species.

Fig. 1 Sensitivity and specificity of LAT, Dot-ELISA and EITB with faecal supernatant antigen of *E. granulosus*

Fig. 2 Latex agglutination test showing positive reaction for *E. granulosus* with faecal supernatant antigen

Fig. 3 Dot-ELISA showing positive reaction for *E. granulosus* with faecal supernatant antigen

The study conducted by Gasser et al. (1988) demonstrated that 25–60 % of the serum from dogs infected with *E. granulosus* did not show significant levels of specific antibody and revealed cross reactivity with other parasite species. Similarly Jenkins et al. (1990) evaluated somatic antigen of *E. granulosus* for sero-diagnostic purpose and found variable diagnostic sensitivity and high cross reactivity with antigens from other parasitic species. The latex agglutination test is a rapid, easy to perform and required only 3–5 min for completion. The test can be used to screen large number of samples in a short period of time to ascertain the sero-prevalence of the disease.

Fig. 4 Known positive sera of faecal supernatant antigen of *E. granulosus* showing immunoreactive peptides

Among 250 serum samples of stray dogs examined for the detection *E. granulosus* specific antibodies in dogs by Dot-ELISA with faecal supernatant antigen, 45 (18 %) serum samples were found positive (Fig. 3). The sensitivity and specificity was found to be 100 and 82.66 %, respectively (Fig. 1). This visually read enzyme immuno assay is rapid, portable, reagent conservative, with high sensitivity and specificity. Furusawa (1997) used a Dot-ELISA, to visualize the presence of the copro-antigens for ‘on the spot’ diagnosis of *E. multilocularis* infection in definitive hosts and observed that the assay was very much suitable for field surveys. This was an original attempt on serodiagnosis in the detection of *E. granulosus* infection by Dot-ELISA with F/S antigen of *E. granulosus*. The Dot-ELISA test is very simple to perform and large number of serum samples can be screened in short time. The test was considered useful for the detection of echinococcosis in dogs in naturally infected conditions at field level.

Out of 250 serum samples examined for *E. granulosus* infection in dogs by EITB with fecal supernatant antigen, specific antibodies were detected in 47 (18.80 %). The immune-reactive polypeptides of 45 and 34 kDa were detected in all the positive serum samples (Fig. 4). The sensitivity and specificity was found to be 100 and 86.80 %, respectively (Fig. 1). Recently, Guarnera et al. (2000) found the sensitivity and specificity of copro-western blot with F/S antigen to be 70 and 100 % respectively. Interestingly, the higher sensitivity observed in the present study might be due to the type of antigen used for detection.

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