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Immunodiagnosis of *Echinococcus granulosus* Infection in Dogs by Indirect-Enzyme Linked Immunosorbent Assay

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Abstract: The present study was undertaken for the detection of *Echinococcus granulosus* specific antibodies in dogs by indirect-ezyme linked immunosorbent assay. Two different antigens viz., somatic and excretory secretory (E/S) antigens were prepared from *E.granulosus* adult worm. Totally 250 field dog serum samples were examined for the detection of anti-*E.granulosus* antibodies using somatic and excretory secretory antigens of *E.granulosus*. The specific antibodies of *E.granulosus* to somatic and excretory secretory antigen were detected in 62 (24.8 per cent) and 81 (32.4 per cent) dog serum samples by indirect ELISA. The sensitivity and specificity of indirect ELISA with somatic antigen was found to be 100 and 71.60 per cent respectively. However the sensitivity and specificity of indirect ELISA with excretory secretory antigen was found to be 100 and 79.20 per cent. It was concluded that the indirect-ELISA can be effectively used for specific detection of *Echinococcus granulosus* infection in dogs and can be used to ascertain the actual status of the *E.granulosus* infection in dogs in an endemic area.

Key words: Indirect ELISA, *E.granulosus,* Dog, Somatic antigen, E/S antigen

INTRODUCTION

Echinococcus granulosus, the dwarf tapeworm of dog, is the causative agent of cystic echinococcosis in domestic animals and man, an important zoonotic disease widely distributed throughout the world. Echinococcosis in dogs cannot be differentiated from other taeniosis by using conventional parasitological techniques and morphological identification is not reliable and confirmatory based on coproscopic examination [1].

This parasite is distributed worldwide but is more common in areas where hygienic conditions are poor and literacy is low. The role of uncontrolled stray dog population especially in underdeveloped countries need not be overstressed. There is enough evidence to show that the disease is spreading because of a lack of appropriate legislation on animal slaughter, dog management and sanitary facilities [2]. *E.granulosus* is an obligatory heterogeneous parasite with a complex life cycle. It requires two mammalian hosts to complete its life cycle. This involves the definitive hosts (domestic dogs and wild canids) and the intermediate hosts (domestic and wild ungulates and human beings) [3].

In India, a high prevalence of cystic echinococcosis has been reported in man as well as in livestock. The socio-economic, cultural and religious factors have frequently played an important role in the transmission of infection to human beings. While much attention has been focused on the prevalence, diagnosis and epidemiology of cystic echinococcosis, less attention has been focused on *E.granulosus* infection in dogs.

The development of sensitive and specific ante-mortem diagnostic methods for the detection of canine echinococcosis is important for the 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 per cent, it is time-consuming, biohazardous, has variable sensitivity and requires trained personnel [4].

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In India, both immunological and molecular techniques have been standardized for diagnosis of cystic echinococcosis in intermediate hosts. But even though dogs are the main disseminators of infection as definitive hosts, very few studies have been undertaken to observe the prevalence of echinococcosis. The scanty reports are mainly based on post mortem observation. Therefore the present study was undertaken f or the detection of *E. granulosus* specific antibodies in dogs by indirect-ezyme linked immunosorbent assay.

MATERIALS AND METHODS

Preparation of Somatic Antigen: The somatic antigen was prepared by following the procedure of Elayoubi and Craig [5] with slight modification. The worms recovered from the small intestine of dogs were washed thoroughly in Hank's balanced salt solution. Immature segments of about 500 worms were separated and transferred to a screw capped vial containing 0.15 M phosphate buffered saline (pH 7.2). The segments were triturated using a glass mortar and pestle. The contents were repeatedly frozen and thawed four times and then disrupted by Sonirep 150 ultrasonication (Sanyo Gallenkamp PLC, UK) three times for 20 seconds each time at 100 mAmp and less than 4°C. The suspension was centrifuged at 12000 rpm for 30 minutes in a refrigerated centrifuge (4°C) (Superspin). The supernatant was collected and used as the soluble antigen extract. The protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) (Sigma, USA) was added at concentration of 2 µl/ml of antigen. The antigen was aliquoted and stored at -20°C till further use.

Preparation of Excretory Secretory Antigen: The E/S antigen was prepared as per the methodology of Elayoubi and Craig [5] with slight modification. The small intestine of dogs found positive for *E.granulosus* were divided into several equal parts, opened and placed over a mesh in a Petri dish with the mucosal surface in Hank's balanced salt solution (HBSS) (Himedia, Mumbai) and incubated for 1 hour during which the adult worms were released from the gut mucosa. The worms which were recovered were thoroughly washed with HBSS (pH 7.2) containing gentamicin (200 µg/mL). The worms were transferred to Medium-199 (Himedia, Mumbai) pH 7.2 supplemented with glucose (4.0 g/L) and gentamicin (200 µg/ml) and maintained at 37°C with 5 % Co_2 concentration in the incubator (Sanyo). Approximately 500 worms were cultured in 10 mL of medium. The medium was replaced every 6 hours during the first 24 hours, pooled and stored at -20 °C until processed. The medium in aliquots of 10 mL containing the E/S components was transferred to a dialysis tube and dialyzed against PBS and then concentrated using poly ethylene glycol (PEG) (Himedia, Mumbai) followed by dialysis with PBS. The dialyzed fraction was subjected to centrifugation at 12,000 rpm for 30 minutes in a refrigerated centrifuge. The supernatant was collected and PMSF was added at the concentration of 2 µL per ml of antigen. The excretory/secretory antigen was stored at –20°C in aliquots.

Estimation of Protein Concentration: The protein concentrations of the antigens were estimated as per the method of Bradford [6] using protein estimation kit obtained from Bangalore Genei Co., Bangalore.

Enzyme Linked Immuno Sorbent Assay (ELISA): Indirect ELISA was used to detect antibody of *E.granulosus* as per Allan *et al.* [7] and Ahmed and Nizami [8], respectively. The working dilutions of conjugate, antigen and test sera were determined prior to use by checkerboard titrations.

Determination of Working Strength of Anti-Gog IgG Conjugate: To determine the working dilution of anti-dog conjugate, 100 µL of normal dog serum (1:10000) was coated onto 96 well flat bottom polystyrene ELISA plate (Titertrek) by diluting with coating buffer and incubated at 37 °C for 1 hour. The ELISA plate was washed with washing buffer thrice. The blocking buffer was added to block the non-specific reactive sites and incubated at 37 °C for one hour. The plate was washed with washing buffer thrice. Test conjugate dilutions were prepared in blocking buffer and 100 µL of each dilution was added to the wells in duplicate and incubated at 37 °C for one hour. Further steps were as described in procedure.

Determination of Optimal Serum Dilution: 100 µL of antigens of *E.granulosus* in carbonate buffer was added to 24 wells of a 96 well ELISA plate. The plate was incubated overnight at 4 °C and washed thrice with washing buffer. The blocking buffer was added to block the non-specific reactive sites and incubated at 37 °C for one hour. After washing the plates, 100 µL of positive serum dilutions was added in triplicates and further steps were as described in procedure.

Determination of Cut off Value: Ten known negative serum samples were obtained from a dog breeding unit where the dogs were reared in confinement with no access to infection and were regularly dewormed. This was used to determine the cut off value. The cut off value was

calculated by taking mean absorbance values of known negative sera plus three standard deviation. Any serum with OD values above the cut off value was regarded as positive.

Procedure: The flat bottom polystyrene 96 well ELISA plate was coated with 100 µl containing antigen (2 µg E/S and 3 µg somatic antigen) in coating buffer in duplicates. The plate was incubated at 4 °C overnight and washed thrice with washing buffer. The plates were incubated at 37 °C for one hour after adding 100µl of blocking buffer (5% skimmed milk powder with PBS Tween-20) and washed thrice with PBS Tween-20. The positive serum (1:100 dilution for E/S and 1:200 dilution for somatic antigen) with blocking buffer was added to all wells and incubated for one hour at 37 °C. The plates were washed four times with washing buffer and 100 µL of 1:10,000 diluted anti-dog conjugate was added and incubated as above. The plates were washed five times with washing buffer. Then 100 µL of substrate chromogen working solution was added and color reaction was monitored in dark place. The reaction was stopped by adding 50 µL of 2M H_2S0_4. The absorbance values were read in a Multiscan plus P (Lab systems) ELISA reader at 450 nm. Positive control and negative control was included in the assay in duplicate.

Sensitivity and Specificity of Indirect ELISA: The sensitivity and specificity of Indirect-ELISA was calculated by the following formula:

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

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

Statistical Analysis: The data were analyzed using chi-square test as per the procedure of Daniel [9].

RESULTS

The working dilutions of conjugate, excretory/ secretory antigen and positive serum were found to be 1:10,000, 2 µg/well and 1:100, respectively by checkerboard assay method. The working dilution for somatic antigen of conjugate was 1:10,000; antigen was 3 µg/well and 1:200 dilution for serum.

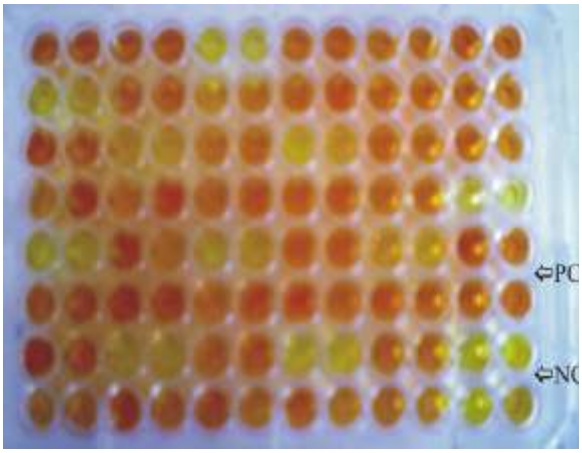

Fig. 1: Indirect-ELISA showing positive reaction for *E.granulosus* with E/S antigen (PC- positive control; NC- negative control)

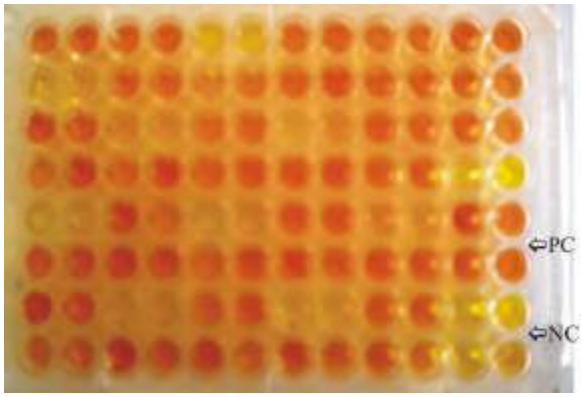

Fig. 2: Indirect ELISA showing positive reaction for *E.granulosus* with somatic antigen (PC- positive control; NC- negative control)

The preliminary assays performed on sera from 10 dogs in which no helminth ova were detected yielded a mean background absorbance value (x) of 0.378 and 0.334 and a standard deviation of 0.036 and 0.031 for E/S and somatic antigen of *E.granulosus,* respectively. The cut off OD value for E/S antigen was 0.486 and for somatic antigen of *E.granulosus* was 0.427 (Mean ± 3 SD).

In the present study indirect ELISA was conducted for detection of serum antibodies in dogs, specific to *E.granulosus* with E/S and somatic antigen. The E/S antigen of *E.granulosus* was detected in 32.4 % out of the 250 field serum samples examined (Fig. 1).

All negative serum controls were negative in the assay and the OD values of positive serum ranged from 0.486 to 0.760 and are depicted in the graph (Fig. 3). Out of 250 serum samples examined for presence of

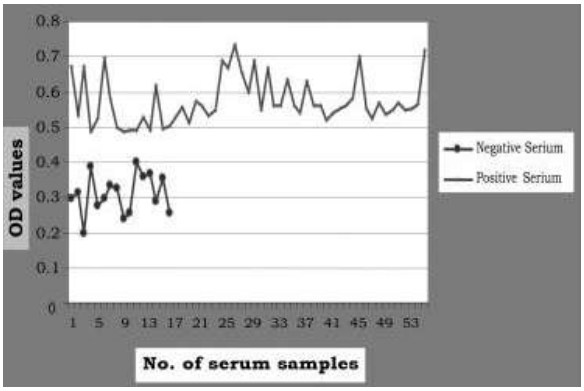

Fig. 3: OD Values of positive and negative serum samples with E/S antigen of *E.granulosus* by ELISA

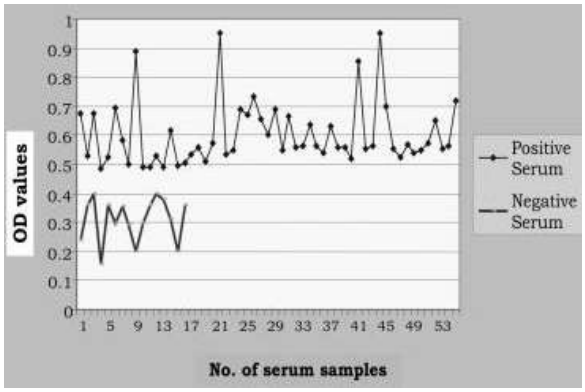

Fig. 4: OD values of positive and negative serum Samples with somatic antigen of *E.granulosus* by ELISA

anti-*E.granulosus* antibodies with somatic antigen in dogs, 24.8 % were found to be positive (Fig. 2). The OD values were in the range of 0.427 to 0.980 (Fig. 4) and the statistical analysis by Chi-square test revealed significant difference between the two antigens (P=0.05).

In the present study cross-reactions were noticed in *Taenia hydatigena* and *Dipylidium caninum* positive samples. The OD values in *E.granulosus* positive dogs were in the range of 0.852 to 1.022. In case of dogs naturally infected with *Taenia* the OD values were 0.336 to 0.754 and for *D.caninum* infected dogs it was 0.348 to 0.689. Since the cut off value was 0.486, the above results with *T.hydatigena* and *D.caninum* were considered as cross reactions.

In the present study, the sensitivity and specificity of ELISA was found to be 100 and 79.20 per cent for E/S antigen and 100 and 71.60 per cent for somatic antigen, respectively. Out of 250 dogs serum samples examined, the false positive reaction was observed in 52 and 71 dogs with E/S and somatic antigen, respectively. On the other hand false negative results were not found against E/S and somatic antigens of *E.granulosus* infected dogs. The high sensitivity was observed due to the heavy worm burden in both the positive samples. The specificity of the ELISA in the present study was found to be lower. There was a difference in the cut off values of 0.486 and 0.427 for E/S and somatic antigen.

DISCUSSION

ELISA has emerged as a very useful immunological tool because of which it became one of the most widely used techniques in measuring antibody, antigen and protein Mclaren *et al.* [10]. In the present study indirect ELISA was conducted for detection of serum antibodies in dogs, specific to *E. granulosus* with E/S and somatic antigen. The E/S antigen of *E.granulosus* was detected in 32.4%. All negative serum controls were negative in the assay. Out of 250 serum samples examined for presence of anti-*E.granulosus* antibodies with somatic antigen in dogs, 24.8%were found to be positive.

Gasser *et al.* [11] used ELISA for the diagnosis of *E. granulosus* infection in dogs with worm excretory/secretory antigen and compared with protoscolex somatic antigen. They tested 224 sera from dogs and found significant linear relationship between absorbance values of the sera tested against the two antigens.

ELISA based serum antibody detection is useful in the diagnosis of naturally aquired *E.granulosus* infection in dogs [12]. Ersfeld *et al.* [13] analyzed adult worm extracts of *E.granulosus* by ELISA and showed a sensitivity of 83 % for cystic echinococcosis. They showed that *E.granulosus* adult worms could provide an alternative source to metacestode antigens for the serodiagnosis of cystic echinococcosis. The study conducted by Zhang *et al*. [14] found poor sensitivity and specificity in ELISA based methods for detection of circulating antibodies in canines and found no correlation of ELISA results with worm burden. According to Carmena *et al*. [15], the excretory-secretory products of *Echinococcus* contain potential diagnostic antigens that can be used in the immunodiagnosis of canine echinococcosis.

In the present study cross-reactions were noticed in *Taenia hydatigena* and *Dipylidium caninum* positive samples. This could be possibly due to sharing of common epitopes/antigenic determinants between the

closely related species. In addition, the antigens used were only partially purified. Studies conducted by Gasser *et al.* [11] demonstrated that 25–60 % of the sera 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*. [16] evaluated somatic antigen of *E.granulosus* for serodiagnostic purpose and found variable diagnostic sensitivity and high cross-reactivity with antigens from other parasite species.

In the present study, the sensitivity and specificity of ELISA was found to be 100 and 79.20 % for E/S antigen and 100 and 71.60% for somatic antigen, respectively. The high sensitivity was observed due to the heavy worm burden in both the positive samples. The specificity of the ELISA in the present study was found to be lower which might have been due to more number of false positive cases. The reduced specificity may also be due to increased background reactivity with sera from dogs infected with *T.hydatigena*. There was a difference in the cut off values of 0.486 and 0.427 for E/S and somatic antigen contrary to 0.330 for worm excretory secretory-ELISA and 0.382 for protoscolex somatic-ELISA reported by Gasser *et al*. [11]. However, they also found a higher specificity of 93.7 % with worm E/S antigen and 97.9 % with protoscolex somatic antigen. This may be attributed to the differences in antigen preparation and type of antigen used.

The excretory secretory (E/S) and somatic antigens of *E.granulosus* are serves as antigens of diagnostic importance. This is the first report where the comparison was made in detection of antibodies by indirect ELISA using E/S and somatic antigens of *E.granulosus*.

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