Immunodiagnosis of *Echinococcus granulosus* infection in dogs by counter immunoelectrophoresis (CIEP)

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Abstract The present study was undertaken for the detection of *Echinococcus granulosus* specific antibodies in dogs by counter immunoelectrophoresis (CIEP) with two different antigens viz., somatic and excretory secretory (E/S) antigens of *E. granulosus* adult worm. Totally 100 field serum samples were examined for the detection of anti-*E. granulosus* antibodies using somatic and E/S antigens of *E. granulosus*. The CIEP can able detect nine and eight *E. granulosus* specific antibodies with E/S and somatic antigen of *E. granulosus*, respectively. The sensitivity was found to be 90 and 80 % and the specificity was 100 % with E/S and somatic antigen of *E. granulosus*, respectively.

Keywords CIEP · *E. granulosus* · Somatic antigen · Excretory secretory antigen

Introduction

Echinococcosis, a cyclozoonotic helminthosis caused by the dwarf dog tapeworm *Echinococcus granulosus* is highly endemic and is considered to be one of the most important parasitic diseases worldwide. This disease is spreading because of a lack of appropriate legislation on animal slaughter, dog management and sanitary facilities (Schwabe 1986). *E. granulosus* is an obligatory heterogeneous parasite with a complex life cycle and requires two

mammalian hosts viz., canids as definitive hosts, ungulates and human beings as intermediate hosts to complete its life cycle (OIE 2004).

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, has variable sensitivity and requires trained personnel (OIE 2004).

Direct examination method has disadvantages as small numbers of worms may be overlooked and parasites consisting of only one or two segments may escape detection (Eckert et al. 2001). Sedimentation and counting techniques (SCT) were found to have sensitivity and specificity nearing 100 % and was considered as the “gold standard” (Eckert 2003; Deplazes et al. 2004). But both techniques are time consuming and labor intensive and further cannot be applied to live animals and alternative techniques are necessary. Hence, a study was undertaken for the detection of *E. granulosus* specific antibodies in dogs by counter immunoelectrophoresis (CIEP) using somatic and E/S antigens of *E. granulosus*.

Materials and methods

Collection of serum samples

Field sera

A total of 100 blood samples were collected from stray dogs live in and around organized slaughter house without

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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 somatic and E/S antigens 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. 10 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 double immuno diffusion (DID) 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 somatic antigen

The somatic antigen was prepared by following the procedure of (Elayoubi and Craig 2004) 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 s each time at 100 mAmp less than 4°C . The suspension was centrifuged at 12,000 rpm for 30 min in a refrigerated centrifuge (4°C) (Superspin). The supernatant was collected and used as soluble antigen extract. The protease inhibitor phenyl methyl sulphonyl fluoride (PMSF) (Sigma, USA) was added at concentration of 2 $\mu\text{l/ml}$ of antigen, aliquoted and stored at -20°C .

Preparation of E/S antigen

The E/S antigen was prepared as per the methodology of (Elayoubi and Craig 2004) 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 h 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 $\mu\text{g/ml}$). The worms were transferred to Medium-199 (Himedia, Mumbai) pH 7.2 supplemented with glucose (4.0 g/l) and gentamicin (200 $\mu\text{g/ml}$) 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 h during the first 24 h, 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 min in a refrigerated centrifuge. The supernatant was collected and PMSF was added at the concentration of 2 μl per ml of antigen and stored at -20°C in aliquots.

Protein estimation

The protein concentration of somatic and E/S antigen was estimated as described (Bradford 1976).

Counter immunoelectrophoresis (CIEP)

The test was carried out as per the method of D'Souza and Hafeez (1999) with slight modifications. Agarose (M/S Himedia, Mumbai) was prepared in tris borate buffer with pH 8.2. Half gram of Agarose was added to 25 ml of tris borate buffer and heated. On to glass slides, five ml of molten buffer gel was poured and allowed to solidify. Two wells of four mm diameter were cut with a well distance of six mm for trials with corresponding hyper immune sera.

To screen serum samples, two rows containing four wells each were cut with a diameter of 4 mm and distance of 6 mm between each well and the bases were sealed with molten agar. The well to be located on the cathodic slide was filled with antigens of *E. granulosus* and other (anodic well) with corresponding HIS for evaluation of the test and later with sera from known positive or negative animal. Electrophoresis was carried out in a suitable trough containing barbitone buffer. The slides were placed on the rack and each end was connected with a filter paper wick dipped in buffer. A current of 50 mA per slide was applied for 90 min. The observations were recorded before and after staining. The slides were then washed in normal saline and stained with Comassie brilliant blue. The sensitivity and specificity of CIEP was calculated by:

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

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

Results

The protein concentration of the E/S antigen and somatic antigen was found to be 540 and 940 µg/ml of antigen respectively. The test was initially standardized using positive serum with E/S and somatic antigen and a clear band could be seen after running the system for 90 min. The test was then evaluated with HIS raised against the E/S antigen and somatic antigen, two clear bands could be seen after running for 90 min. Then the test was performed with field serum (1:2 dilutions).

Ten serum samples from dogs, which were found to be infected with *E. granulosus* worms during necropsy, gave nine positive results against E/S and eight with somatic antigens. The CIEP slides showed one clear band after staining. The dogs, which were heavily infected with *E. granulosus* during necropsy, revealed three bands against HIS. The other positive samples resulted in one band against HIS. The other entire 90 field samples screened showed negative results. No cross-reaction was found when serum positive for *Taenia hydatigena* and *Dipylidium caninum* were used. The sharpness of the band was clearer

Fig. 1 CIEP of somatic and E/S antigen of *E. granulosus* with positive serum

Fig. 2 CIEP of E/S and somatic antigen of *E. granulosus* with HIS

with E/S antigen compared to somatic antigen (Figs. 1, 2). The sensitivity of the test was found to be 90 and 80 % and the specificity was 100 % with both E/S and somatic antigen, respectively.

Discussion

The CIEP was evaluated for the diagnosis of *E. granulosus* infection in dogs using E/S antigen in the present study for the first time. The E/S antigen from heavily positive dog had given a clear band with positive serum. False negative reaction was obtained in the other moderately positive cases. This could be due to the low levels of antibody/antigen used in the assay. Rabbit anti-*E. granulosus* E/S hyperimmune serum with E/S antigen gave positive results. Two bands could be visualized indicating two major antigenic components in the heavily positive sample. This was comparable with the results obtained by Ahmad and Nizami (1998). They obtained a maximum of four precipitin arcs when faecal supernatants of experimentally infected dogs were used against positive dog serum. The somatic antigen of *E. granulosus* also gave promising results with both positive sera and hyperimmune serum. This was in accordance with Katoch and Singh (1994) who used adult worm antigen for detection of echinococcus antibodies in experimentally infected pups by CIEP.

Katoch and Singh (1994) used standard CIEP for detection of echinococcus antibodies in sera of experimentally infected pups on day 20, 30 and 50 post infection using protoscoleces and adult worm antigen. They detected antibodies on day 30 and 50 post infection with adult worm antigen and 20, 30 and 50 days post infection with protoscoleces antigen. Ahmad and Nizami (1998) used CIEP for the detection of coproantigens of *E. granulosus* in dogs on an experimental basis. They used positive dog serum against positive faecal supernatant in the assay. Konapur et al. (1999) used CIEP for diagnosis of hydatidosis in cattle and buffaloes using concentrated crude and partially purified antigens of hydatid cyst fluid, germinal membrane and protoscoleces. The sensitivity and specificity was 90.7 and 88.1 % in cattle and 69.2 and 87.8 % in buffaloes, respectively.

The specificity of CIEP was found to be 100 % in the present study with both E/S and somatic antigen. Sensitivity was found to be 90 and 80 with E/S and somatic antigen, respectively. The high sensitivity and specificity could have been due to the low prevalence of *E. granulosus* by necropsy method, which was taken as the standard for CIEP. Interestingly, in the present study no cross reaction was observed with *T. hydatigena* and *D. caninum*. Tris–borate buffer was used in the present study whereas, Ahmad and Nizami (1998) used barbitone buffer. This was

considered as essential since sodium barbitone was not available.

It can be concluded that, the CIEP was found to be a simple, inexpensive and rapid test. Results can be obtained within 90 min and has a potential for field application since it shows high specificity and sensitivity for both E/S and somatic antigen with positive sera and hyperimmune serum and it was recorded for the first time.

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