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Detection of canine echinococcosis by coproantigen ELISA

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ABSTRACT

Objective: To study the canine echinococcosis by coproantigen ELISA method. **Methods:** During the present investigation experimental infection was established using evaginated worms of *Echinococcus granulosus (E. granulosus)*. To check cross reactivity two pups were infected with *Taenia hydatigena(T. hydatigena)*. In order to detect the presence of antigen, hyperimmune sera were raised against excretory-secretory products of adult worms *E. chinococcus granulosus*. Faecal sample collected either from experimentally infected pups or from other sources were heated at 70 ℃ to detect heat stable soluble antigen. **Results:** Pups harbouring less than 104 worms showed negative results. Samples collected from 14 days onwards from experimentally infected animals harbouring more than 104 worms showed positive value. The maximum positive samples were detected in samples collected from in and around slaughter house and the least number of samples were detected positive maintained by dog squad. **Conclusions:** The affinity purified IgG exhibited promising results for detection of canine echinococcosis by indirect ELISA.

1. Introduction

Echinococcosis is an important zoonosis with worldwide distribution[1]. The parasite life cycle is maintained through dog as definitive host and human and other ungulates as the intermediate host. Accurate detection of the infection in the definitive host is important to develop strategies to reduce the disease transmission.

Different traditional techniques like arecoline purgation, detection of eggs or segments of the adult parasite in the faeces or after necropsy are in use but having some limitation. Detection of eggs in faeces is not reliable as the eggs are morphologically indistinguishable from the other taeniid worms[2] and egg production is also variable. Arecoline purgation and necropsy techniques are time consuming, labour intensive, costly and also hazardous for the operator and the environment.

In the last two decades, different alternative approaches have been developed and evaluated including immunological detection of circulating antibodies[3], specific egg fluorescence[4], coproantigen ELISA[5] and PCR amplification of the parasite DNA from faecal sample[6-7]. But, immunological detection of circulating antibodies exhibited variable sensitivities[8-9] and also unable to differentiate between the present and past infection. DNA based methods are recommended for confirmation of coproantigen test of *Echinococcus multicularis(E. multicularis)*[7]. Lahmar[10] reported that coproantigen ELISA is much more sensitive than that of arecoline purgation and copro-DNA techniques.

Hence, coproantigen ELISA is currently the best laboratory based test for the mass screening of the infection in dogs as it can detect the infection during the prepatent period with a high specificity (>95%) and sensitivity (>90%)[11,12]. Additionally, this technique is less hazardous for the personnel involved as it detects the heat resistant epitopes, possibly carbohydrate moieties of the coproantigen.

In the present communication we have described a method of indirect ELISA using affinity purified IgG of hyperimmune sera which has been correlated with the biomass of the parasite, verified cross reactivity with *Taenia hydatigena(T. hydatigena)* and also screened the samples of domestic and watch dogs as well as stray dogs rescued by non government organization in West Bengal.

2. Materials and methods

This study was designed in two parts. Firstly, coproantigen ELISA was developed and validated exploiting experimental infection in dogs with due permission from the animal ethics committee of the Institution. Secondly, a surveillance study was conducted using the test to estimate the copro-prevalence in owned and stray dogs' different zones of West

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2.1. Collection of protoscoleces and in vitro cultivation of protoscoleces

Hyadatid cysts and *T. hydatigena* metacestodes of buffalo origin were collected from Kolkata municipality abattoir, India. Protoscoleces were collected from fertile cysts and fresh protoscoleces were harvested in M199 medium for evagination at 37 ℃ for a period of twenty four hours as described earlier[13]. The evaginated protoscoleces with viability of 80–90% were used for infection. *T. hydatigena* metacestodes were also maintained in M199 media before infection.

2.2. Experimental infections

A total of 14 pups, irrespective of sex and aged 8 weeks to 10 weeks, of local non descriptive breed were first dewormed using praziquantel (10 mg/kg) and maintained under helminth free environment with adequate food and water for a month before the experimental infection.

Ten pups were infected orally with approximately 5 000 viable protoscoleces and two pups were infected orally with ten *T. hydatigena* cestode. Rest two pups were maintained as negative control. Faecal samples were collected for a period of 42 days at 7 days' interval. All pups were euthanized on 42nd day post infection(DPI).

2.3. Collection of faecal samples for the seroprevalence

A total of 107 faecal samples were collected from different zones of West Bengal, India which is depicted in Table 1 with some specific remarks. The faecal samples were collected either rectally using plastic spatula or from ground just after defaecation with the help of the villagers. The samples were sealed into screw capped storage containers and processed within 24 h. Proper hygienic measures were maintained during collection and processing of the faecal samples.

Table 1
Sources of the faecal samples and coproantigen ELISA result.

Source	Total sample (*n*)	Positive [*n* (%)]	Remarks
Dog squad	19	1 (5.30)	Deworming is done in regular intervals
NGO, Broad Street	18	8 (44.40)	Rescued dogs in and around Kolkata
Midnapore (East & West)	280	134 (47.80)	Stray dogs from villages
South 24 Parganas	170	72 (42.40)	–
Around slaughter houses, Kolkata	253	143 (56.50)	Stray dogs
Total	740	358 (48.37)	–

2.4. Preparation of faecal samples

Collected faecal samples were processed as per methodology described earlier[14]. The collected faecal samples were mixed in 1:4 ratios (w/v) with 1 percent formalin. The mixture was heated at 70 ℃ for a period of 12 h at 2 200 g for 10 min. The supernatant was collected and stored at −20 ℃ till further use.

2.5. Recovery of the worms and production of E/S products

Adult worms were recovered from the small intestine of the infected dogs at 42nd DPI. Briefly, small intestine was cut longitudinally and placed in a petri dish containing Hank's balanced salt solution (HBSS, pH 7.2) and the biomass was recorded for the individual dog. Worms were then washed thrice in HBSS (pH 7.2) containing gentamicin (200 μg/mL). Approximately, 1 500 of viable worms were cultivated per 50 mL of M199 (Hi Media, India) medium supplemented with glucose (4 mg/mL) and gentamicin (200 μg/mL) and incubated at 37 ℃ with 5% CO_2 tension. Medium was replaced every 6 h interval during 24 h incubation and stored at −80 ℃ until processed. The medium containing the E/S components was concentrated using dialysis against PBS (pH 7.2). Protein concentration of the E/S product was estimated by Bradford assay[15].

2.6. Preparation of anti– E granulosus E/S polyclonal antibody

For the preparation of the polyclonal antibody, rabbits were immunized using 100 μg of the E/S product alongwith Freund's complete adjuvant as described[14]. Freund's incomplete adjuvant was used for the second and third inoculation two weeks apart. IgG was purified from 10 mL of the pooled sera using GeNeiTM IgG Purification Kit (Bangalore Genei, India). Affinity purified IgG was dissolved in 10 mL of PBS (pH7.2).

2.7. Enzyme linked immuno sorbent assay

ELISA for coproantigen detection was performed following the protocol described[16]. Flat bottomed polystyrene plates (Polysorp Immuno Plate, Nunc, Denmark) were coated with preserved coproantigen samples and incubated overnight at 4 ℃. Next day after washing with PBS–T (PBS with 0.05% Tween20) the plate was blocked with 2% BSA in PBS (pH 7.2) for 2 h at 37 ℃. Purified IgG was added at 1:100 dilutions and incubated at 37 ℃ for one hour. After washing, goat anti rabbit IgG HRP (Genei, India) conjugate was used at 1: 10 000 dilutions. Colour development was done using O–phenylenediamine (OPD) (SIGMAFAST™ OPD, Sigma, USA) and the reaction was stopped using 0.1 M sulphuric acid. Optical density (OD) was measured at 492 nm. The considered value of A_{492} represents the mean of two readings. For the interpretation of seropositivity, threshold values (cut–off) were calculated: mean OD of negative sera samples ± 3SD.

For the seroprevalence study, three samples each from the post mortem confirmed positive and negative dogs were taken as reference sample in ELISA.

2.8. Statistical analysis

The data of the experimental infection were analyzed (two way ANOVA) using GraphPad Prism version 4.00, GraphPad Software, San Diego California USA.

3. Results

After necropsy at 42nd DPI, experimentally infected dogs were harbouring different numbers of adult parasites in a range of 68-3020 worms (Figure 1 & 2).

Protein profile of the whole antiserum raised against the E/S product of the adult parasites and the purified IgG has been depicted in the Figure 3. The relative molecular weight of affinity purified IgG following denaturation was within 55-70 kDa.

Cut-off value of the ELISA was 0.082. OD value above 0.082 was considered as positive sample. The result of coproantigen ELISA of the individual experimentally infected dog with the respective biomass in different time course is depicted in Figure 1 and Figure 2 and the differences found to be significant ($P<0.01$).

At the 28th DPI the OD value of the dogs are positively correlated with the corresponding biomass (at 95% confidence interval, $R^2= 0.8755$) which is depicted in Figure 4.

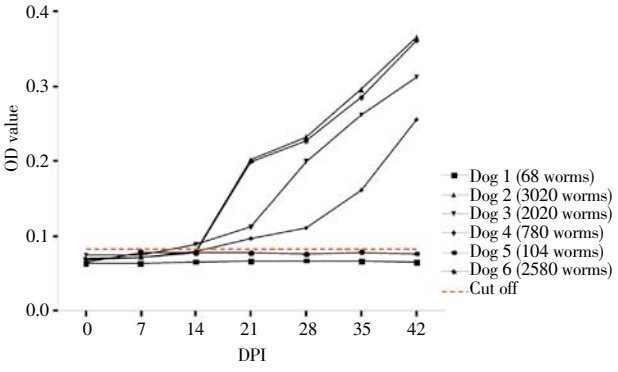

Figure 1. Showing OD values at different time interval correlated with biomass of the parasite.

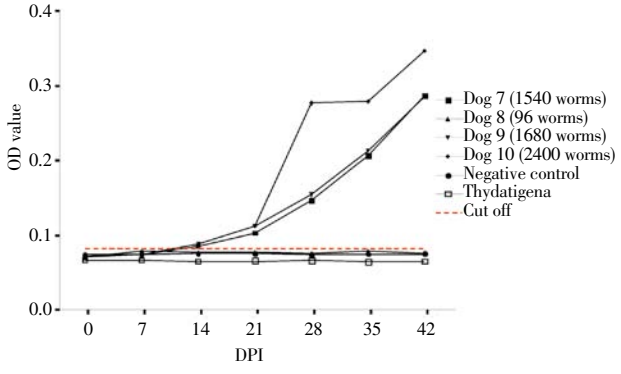

Figure 2. Showing OD values at different time interval correlated with biomass of the parasite, negative control and *T. hydatigena* infected dogs.

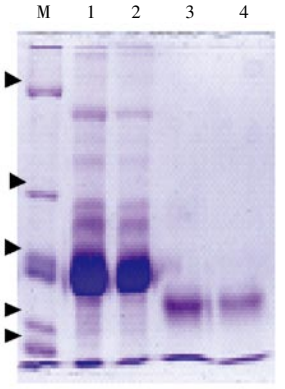

Figure 3. Relative migration of whole serum samples (lane 1 & 2), affinity purified samples (lane 3 & 4) and M indicates marker (arrows from the bottom 29, 43, 66, 97.4 and 205 kDa).

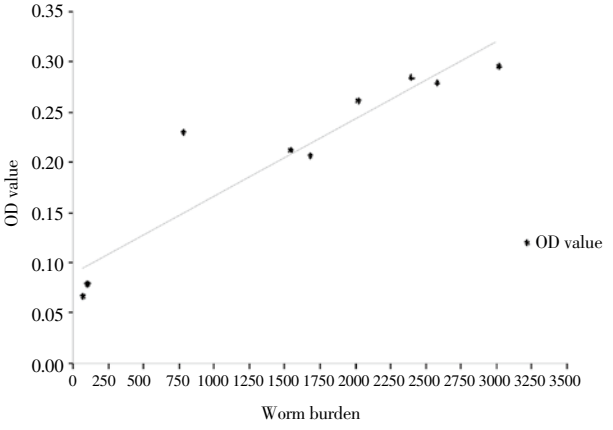

Figure 4. Correlation of biomass and worm burden.

From the 14 DPI onwards all the dogs except dog 1, dog 5 and dog 8 were positive and none of the dogs of the negative control group showed false positive reaction. There was no rise in OD value of the dog1, dog 5 and dog8 till the last day of the experiment (42 DPI).

Faecal samples from the *T. hydatigena* infected dogs were assayed in different time course which always remained below the cut-off value (Figure 1 & 2).

A total of 740 faecal samples were collected from different sources. Out of which 358 (48.37%) samples were positive for the infection in West Bengal. Prevalence data in respect of the sources showed a great variation (range 5.3%-56.5%) which is depicted in Table 1.

Faecal samples from the dog squad where the dogs are regularly de-wormed showed the least (5.3%) and the stray dogs around the slaughter house and meat markets in Kolkata showed the highest prevalence of the disease (56.5%).

4. Discussion

Identification of canine infection is a critical requirement

to establish epidemiological status of cystic echinococcosis in an area. Immunodetection of heat stable soluble antigens in faecal material of the infected dogs has gained increasing interest as an alternative[17]. Here, we have standardized one coproantigen based ELISA and used it for generation of prevalence data.

In this study, we have used affinity purified IgG fraction of the rabbit anti-Px-ES antisera to enhance the sensitivity of the assay. Overnight heating of the faecal material along with 1% formalin reduces the risk of infection to the persons involved, without altering the properties of the coproantigens[18].

Sensitivity of the coproantigen ELISA is critically dependent on the parasite burden[5,14]. In the present study, false negative results were seen in all the dogs harboring less than 104 worms throughout the study. Rest of the dogs harboring higher burden were positive after 14th DPI. The OD values of the true positive dogs are in positive correlation with the corresponding worm burden. The OD values of the dogs with lesser worm burden came closer to those of the dogs with higher worm burden towards the end of the study.

This indicates that the antigens released are related to the biomass of the parasite. It may also be due to the stage specific excretion of the antigen by the growing parasite. These data are well corroborated with the study by Casaravilla[14].

In our study, dogs infected with *T. hydatigena* showed no cross reaction as the OD values always remained below the cut-off value throughout the study.

From the copro-prevalence study, it can be concluded that prevalence of canine echinococcosis is more in canines residing in and around slaughterhouses, Kolkata. Stray dogs of the rural areas in the two districts of West Bengal are also with high infection rate. The least prevalence of canine echinococcosis in dogs maintained in dog squad, Broad Street speaks about the regular deworming in the dogs.

From our study, it can be concluded that coproantigen ELISA can be used as a valuable method for the surveillance of the infection in canine population.

Conflict of interest statement

We declare that we have no conflict of interest.

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