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Diagnosis of *Echinococcus granulosus* infection in dogs by a coproantigen sandwich ELISA

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ABSTRACT

Echinococcosis is a near-cosmopolitan zoonosis caused by the adult or larval stages of tapeworms belonging to the genus *Echinococcus*. Cystic echinococcosis in food animals is highly prevalent in India in general and in the Karnataka state in particular. A sandwich ELISA was standardized and evaluated in field conditions for coproantigen detection of *Echinococcus granulosus* infection in dogs. Field fecal supernatants were titrated against positive dog serum. A total of 368 fecal supernatants were tested and 16 samples were found to be positive in sandwich ELISA. Sensitivity and specificity was calculated by taking necropsy as the standard. The sensitivity was found to be 100 percent and the specificity was 96.94 percent.

Key words: *Echinococcus*, coproantigen, dog, fecal supernatant, sandwich ELISA

Introduction

Parasitic diseases with zoonotic potential present an important public health problem and are also of major socioeconomic importance worldwide. The genus *Echinococcus* consists of five species which are presently recognized as *Echinococcus granulosus, Echinococcus multilocularis, Echinococcus oligarthus* and *Echinococcus vogeli* (THOMPSON and LYMBERY, 1995) and the new species *Echinococcus shiquicus* (XIAO et al., 2006). On a global basis, *Echinococcus granulosus* is the most common species which is most widespread with endemic foci on every inhabited continent. Foci of hydatid disease exist in India and the highest prevalence has been reported in Andhra Pradesh and Tamil Nadu compared with other parts of the country (NEPALIA et al., 2006).

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Accurate diagnosis of *Echinococcus* infection in definitive hosts had always been an important component for establishing epidemiological parameters of echinococcosis and preventing human and livestock infection (SAKAI, 1996). The diagnosis of intestinal *Echinococcus granulosus* infection in living dogs is difficult because the small proglottids spontaneously discharged with feces are usually overlooked. Routine coprological techniques cannot differentiate the eggs of *Echinococcus* from other *Taenia* species due to extreme morphologic similarity (DINKEL et al., 1998). Two major diagnostic methods have been extensively used in dogs, purgation with arecoline compounds and necropsy of the small intestine (UNRUH et al., 1973; CRAIG et al., 1995). Necropsy is the method of choice and is considered as the gold standard, but both the methods have many limitations (JENKINS et al., 2000; LOPERA et al., 2003).

Alternatively, immunodiagnostic techniques were used to detect specific antibodies or antigens. During the last three decades, considerable progress has been achieved in various fields of echinococcosis research. Several immunological and serological tests have been evolved for the diagnosis of *Echinococcus* spp.in definitive hosts. Coproantigen detection appears to be valuable in detecting infection in a definitive host with high specificity and sensitivity. ALLAN et al. (2003) defined parasite coproantigens as parasite specific products in the feces of the host that are amenable to immunological detection and are associated with parasite metabolism. They should be present independent of parasite reproductive material (i.e. taeniid eggs or proglottids) and disappear from feces shortly after removal of the intestinal infection. The detection of parasite specific antigens in host feces was first reported for canine *Echinococcus granulosus* by BABOS and NEMETH (1962). Arecoline tests or coproantigen tests with fecal matter obtained directly from the dog contribute information on individual prevalence, while the use of coproantigens detected in ground collected samples is of greater epidemiological value (CAVAGION et al., 2005).

Hydatidosis in food animals is highly prevalent in India in general and in Karnataka in particular. Studies based on slaughterhouse material have indicated the prevalence ranging from 3.02% to 28.26% in five species of food animals. Conversely the actual status of *Echinococcus granulosus* infection in the definitive host is not known although a few attempts have been made to note the prevalence of gastrointestinal parasites in dogs. So in this study a sandwich ELISA was standardized and evaluated for the diagnosis of *Echinococcus* infection in dogs in the Bangalore urban district.

Materials and methods

The study was designed to standardize and evaluate sandwich ELISA for the detection of *E. granulosus* coproantigens in the fecal material of dogs collected from various parts of the Bangalore urban district.

The dogs which were necropsied in the department of Pathology, Veterinary College, Bangalore and also those which died of natural causes in different animal shelters formed the main material for the study. Fecal samples were collected from clinical cases of Veterinary Hospitals in Bangalore district and from stray dogs at animal shelters in different parts of Bangalore.

Adult *Echinococcus* worms recovered from necropsied dogs materials were used for the preparation of excretory/secretory antigen as per MALGOR et al. (1997). Approximately 500 worms were cultivated in 10 mL of Medium 199, pH 7.2 supplemented with glucose (4.0 g/L) and gentamicin (200 μg/mL) and maintained at 37 ^{0}C with 5% CO2 in the incubator. The medium was replaced every 6 hours during the first 24 hours, collected, and stored at -20 ^{0}C until processed. The medium in aliquots of 10 mL containing the E/S components was dialyzed against PBS and then concentrated using polyethylene glycol (PEG). The dialyzed fraction was subjected to centrifugation at 12,000 rpm for 30 minutes in a refrigerated centrifuge. The supernatant was collected and phenyl methyl sulphonyl fluoride (PMSF) was added at concentrations of 2 μL/mL. The excretory/secretory antigen was stored at -20 ^{0}C in aliquots. The protein concentrations of excretory/secretory antigens were estimated as per the method of BRADFORD (1976).

Hyperimmune serum was raised in two healthy rabbits against the excretory/secretory antigen as per standard protocol. Agar gel precipitation test (AGPT) was done with 0.8% agarose in saline and Counter immunoelectrophoresis (CIEP) was carried out using 2% agarose with tris borate buffer, pH 8.0 at a constant current of 8 mA per slide for 60 minutes. After electrophoresis the slides were fixed and stained with Coomassie brilliant blue stain.

IgG from hyper immune serum was purified using IgG purification kit based on protein A affinity chromatography procured from Bangalore Genei Co., Bangalore. Purified IgG from hyper immune serum against excretory/secretory antigen of *Echinococcus granulosus* was conjugated to horse radish peroxidase (HRP) using HRP conjugation kit from Bangalore Genei Co., Bangalore.

Two grams of faecal sample were mixed (1:2) with phosphate buffered saline (pH 7.2) containing 0.3% Tween 20 (PBS-T). It was vigorously mixed in a 15 mL centrifuge tube until slurry was formed. The fecal slurry was centrifuged at 2000 rpm for 20 min at room temperature. The supernatant was collected into a 2 mL screwcapped tube, to which was added 2 μL/mL of PMSF and was labeled with the reference number and stored at -20 ^{0}C until used for coproantigen sandwich ELISA.

Sandwich ELISA was standardized and copro ELISA test was performed by standard technique as per ALLAN et al. (1992). 100 μL rabbit anti *Echinococcus granulosus* ES IgG (1:100) in carbonate buffer was coated on to wells of a 96 well flat bottom polystyrene

ELISA plate (Titertrek). The plate was incubated overnight at 4 ^{0}C and washed thrice (3×5 min) with washing buffer. The blocking buffer was added to block the non-specific reactive sites and incubated at 37 ^{0}C for one hour. After washing the plates, 100 µL of test fecal supernatants in 0.15 M Phosphate Buffered Saline (pH 7.2) plus 0.3% Tween 20 (PBS-T) was added in duplicates. The ELISA plate was washed with washing buffer thrice (3×5 min). 100 µL of rabbit anti *Echinococcus granulosus* ES IgG conjugate (1:200) in blocking buffer was added to the wells and incubated at 37 ^{0}C for one hour. The plates were washed five times (5×5 min) with washing buffer. Then 100 µL of substrate chromogen working solution was added and color reaction was monitored in a dark place. The reaction was stopped by adding 50 µL of 2 M 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 maintained in duplicate. The sensitivity and specificity of ELISA was calculated by taking necropsy as the standard.

Results and discussion

The protein concentration of the excretory/secretory antigen was found to be 450 µg/mL of antigen. The excretory/secretory antigen with hyper immune serum gave good results in AGPT and CIEP. The IgG purified from hyper immune serum against ES antigen gave good results in CIEP. OD values above 1.5 were obtained in 1:200 dilution of conjugate when titrated against 1:2 dilution of positive fecal supernatant at 450 nm by ELISA.

The working dilutions of detecting antibody (rabbit anti *Echinococus granulosus* ES IgG conjugate) and capture antibody (rabbit anti *Echinococus granulosus* ES IgG) were found to be 1:200 and 1:100 respectively by checkerboard assay method. Positive fecal supernatant in 1:2 dilutions were used for the titration. The cut off value was calculated with 10 known negative dog fecal supernatant and the cut off OD value was determined as 0.402 (Mean + 3 SD).

Sandwich ELISA is currently the best overall laboratory based test for ante mortem diagnosis of canine echinococcosis (ECKERT et al., 2001). DEPLAZES and ECKERT (1996) reported sandwich ELISA as a useful technique for field application and a replacement for parasite detection at necropsy. FRASER and CRAIG (1997) reported high genus specificity with polyclonal antibodies, even when crude somatic proglottid extracts were used. DEPLAZES et al. (1992) developed coproantigen detection ELISA tests that use polyclonal antibodies to *Echinococcus granulosus* excretory/secretory (ES) antigens. Sandwich ELISA is highly specific and it detects immature and mature stages of *Echinococcus* spp.and is correlated with the worm burden and the duration of the infection (CRAIG et al., 1995; AHMAD and NIZAMI, 1998). THEVENET et al. (2003) opined that the presence of antigens was independent of the condition of the feces (fresh or dried). Coproantigens

are highly stable in the environment for 6 days and up to 1 year under storage at -20 ºC (JENKINS et al., 2000). The very stable carbohydrate rich molecules in coproantigens retained the antigenicity in the gut and fecal environment (FRASER and CRAIG, 1997).

In this study, a double-antibody sandwich ELISA was used for the detection of *E. granulosus* excretory/secretory products in feces for the immunodiagnosis of intestinal echinococcosis in dogs. The assay sensitivity was enhanced by utilizing the purified rabbit IgG fraction of anti *E. granulosus* excretory/secretory (ES) antigen as capture antibody and HRP conjugated anti *E. granulosus* excretory/secretory (ES) IgG as detecting antibody. In an attempt to improve the ELISA sensitivity, faecal supernatants were assayed at 1:2 dilutions, more concentrated than those used by other authors (DEPLAZES et al., 1992; AHMAD and NIZAMI, 1998) but similar dilution as BENITO and CARMENA (2005). A total of 368 fecal supernatants were tested. Those included two fecal supernatants positive for *Echinococcus granulosus,* 8 positive for *Taenia* spp, 71 positive for *Dipylidium caninum*, 19 supernatants positive for other parasites that were determined during necropsy and 268 field samples. Of them 16 samples were found to be positive in sandwich ELISA. The OD values of positive faecal supernatant were in the range of 0.420 to 0.836 and are depicted in the graph (Fig. 1). All negative fecal controls were negative in the assay.

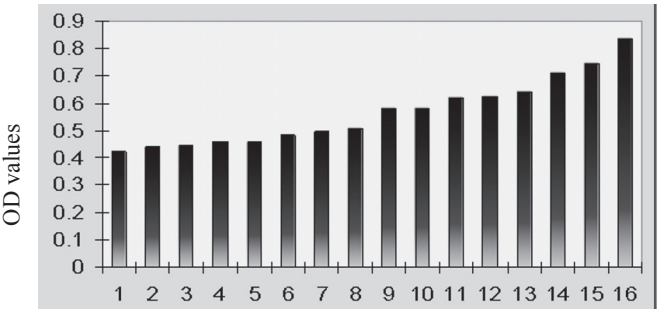

Fig. 1. OD values of positive fecal supernatants detected by sandwich ELISA

A prevalence of 4.35% was obtained by sandwich ELISA during the present study. Higher prevalence rates of *Echinococcus granulosus* infection in dogs have been reported in the following studies: CRAIG et al. (1995) observed 22.7% prevalence of *Echinococcus granulosus* with sandwich ELISA test in Uruguay. MORO et al. (1999) reported 46%, LOPERA et al. (2003) 82%, and MORO et al. (2005) 51% prevalence of *Echinococcus granulosus* infection in Peru by sandwich ELISA test. SVOBODOVA and LENSKA (2002) found 8.1% prevalence of echinococcosis in dogs in the Czech Republic by coproantigen ELISA test. BUISHI et al. (2005) reported a 21.6% prevalence of *Echinococcus granulosus*

in stray dogs in Libya. CAVAGION et al. (2005) in Argentina found 7.3% prevalence of echinococcosis in dogs by sandwich ELISA test. However CHRISTOFI et al. (2002) observed 0.2% prevalence of *Echinococcus granulosus* infection in dogs of Cyprus.

The two positive fecal samples from dogs in the present study showed positive results and cross reactions were less when compared to that of indirect ELISA. Cross reactions were found with 2 out of 8 *Taenia* positive and 1 out of 71 *Dipylidium* positive fecal supernatants. Out of the 268 field fecal supernatants tested, 11 revealed positive OD values. All the positive fecal supernatants identified by sandwich ELISA were retested and one sample showed a negative result. The sensitivity was found to be 100 percent and the specificity was 96.94 percent.

In the present study sandwich ELISA was found to be highly specific in the detection of *Echinococcus granulosus* coproantigens. Significant crossreactions have not been observed with known *Taenia* spp. positive or *Dipylidium caninum* positive fecal supernatants. Cross-reactivity with *Taenia* spp. was observed in the previous studies also (CHRISTOFI et al., 2002; LOPERA et al., 2003; CASARAVILLA et al., 2005). Overall specificity was found to be 97%. Similar degrees of specificity were reported by ALLAN et al. (1990) and MALGOR et al. (1997). The specificity and sensitivity observed in the present study was comparable with COHEN et al. (1998) who reported high specificity (>95%) and sensitivity (100%) in sandwich ELISA with worm counts greater than 20.

The prevalence of 4.35% obtained by this test indicated the prevalence rate of *Echinococcus granulosus* in stray dogs in the Bangalore urban district. It was concluded that this test is a valuable tool for the immunodiagnosis of intestinal echinoccocosis in dogs, allowing its use in surveillance and control programs of this zoonosis.

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SAŽETAK

Ehinokokoza je zoonoza proširena diljem svijeta, uzrokovana odraslim i larvalnim oblicima trakavica roda *Echinococcus*. Cistična ehinokokoza u farmskih životinja vrlo je česta u Indiji, i to osobito u državi Karnataka. Standardiziran je sendvič imunoenzimni test te je ocijenjena njegova vrijednost u terenskim uvjetima u cilju otkrivanja koproantigena podrijetlom iz trakavice *Echinococcus granulosus* u pasa. Nadtalog suspenzije izmeta titriran je u odnosu na pozitivan uzorak pasjega seruma. Od 368 uzoraka nadtaloga izmeta, koproantigeni su bili dokazani u 16 uzoraka. Osjetljivost i specifičnost testa određene su usporedbom s razudbenim nalazom i nalazom trakavica. Dokazano je da test otkriva sve invadirane životinje. Osjetljivost testa iznosila je 96,94%.

Ključne riječi: *Echinococcus*, koproantigen, pas, nadtalog izmeta, sendvič imunoenzimni test