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Standardization of recombinant *Ancylostoma caninum* cysteine protease 2 (rAcCP2) based indirect ELISA for serodiagnosis of hookworm infection in dogs

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ABSTRACT

Diagnosis of hookworm infection in dogs during pre-patentency or in pregnant bitches harbouring encysted larvae in tissues can be achieved by employing serological tests using proteins derived from somatic or excretory-secretory products of adult or larvae of *Ancylostoma caninum*. In the present study, cathepsin-B protease (AcCP2) of *A. caninum*, which helps in development of parasitism and nutrient digestion, was used to develop an indirect ELISA for detection of specific antibodies to *A. caninum* in dogs. The rAcCP2 (approx. 59.0 kDa) was cloned, expressed and purified under denaturing conditions. Serum samples of 20 known *A. caninum* positive and 15 known negative dogs were used for the standardization of indirect ELISA. The sensitivity and specificity of the assay was determined by using sera samples from 123 dogs (48 positive for *A. caninum* eggs in faeces and 75 faecal negative). Out of the 48 *A. caninum* faecal positive sera, 46 were tested positive (OD > 0.306) by indirect ELISA; whereas, 14 out of 75 faecal negative samples were also tested positive (OD > 0.306) by indirect ELISA. Based on the results, the sensitivity and specificity of rAcCP2 based indirect ELISA was calculated to be 95.8% and 84.3%, respectively. No cross-reactions were observed with serum from dogs naturally infected with *B. canis vogeli*, *B. gibsoni*, *E. canis*, *Dirofilaria immitis* and *Toxocara canis*. The results of the present study indicate that indirect ELISA with rAcCP2 protein might be appropriate in large scale epidemiological screening and for serological diagnosis of *A. caninum* infection in dogs.

Key words: *Ancylostoma caninum*, Indirect ELISA, rAcCP2, Serodiagnosis

Ancylostoma caninum, the canine hookworm, is one of the most pathogenic helminths of dogs causing iron deficiency anemia and bloody, wine-dark or tar-black diarrhoea. Larvae and adults of *Ancylostoma* spp. are also involved in human infections, resulting into cutaneous larva migrans or creeping eruptions and eosinophilic enteritis. Diagnosis of hookworm infection is routinely made by demonstration of eggs in faeces. However, during prepatent infection, faecal examination may turn negative. Also, most dogs especially, pregnant bitches, who recover from hookworm infection, become carriers as larvae become encysted in their tissues. Diagnosis of infection in such cases cannot be achieved by routine parasitological methods. Therefore, it is important to diagnose the infection in prepatency or in carrier animals using serological techniques. Presently, the serological diagnosis of hookworm infection is mainly based on use of native proteins derived from somatic or excretory-secretory products of adult or third stage larvae of *A. caninum* (Bungiro and Cappello 2005). There is an urgent need of an immunodiagnostic test based on use of recombinant proteins, as the harvesting of native proteins is difficult, cumbersome and results vary from batch to batch.

Hookworm proteins involved in tissue invasion process are particularly good candidate antigens for development of diagnostics. Cysteine proteases from parasitic helminths, belong to a wide range of protease family known as cathepsin B like proteases (Skuce *et al*. 1999) and are pivotal for parasitic existence, mediating fundamental physiological processes such as moulting, tissue invasion, feeding, embryogenesis and evasion of host immune response. Cathepsin B protease activity has been reported to be present in the adult, larvae and eggs of *A. caninum* (Harrop *et al*. 1995a, b, Yang *et al*. 2011). The immunolocalization of cathepsin B proteinase (AcCP2) in eggs and larval stages of *A. caninum* suggests that these proteases, besides helping in development of parasitism and nutrient digestion, also relate to the embryo and larval development (Yang *et al*. 2011).

Keeping the above facts in mind, the present study was envisaged to evaluate the use of recombinant cathepsin-B

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protease (AcCP-2) of *A. caninum* in indirect-ELISA format for the immunodiagnosis of *A. caninum* infection in dogs.

MATERIALS AND METHODS

Collection of adult Ancylostoma caninum*:* Live *Ancylostoma caninum* adult worms were collected during post-mortem examination of a dog that had died during a road accident. The live adult parasites were identified, washed three times in PBS (pH-7.2) and finally kept in RNALater at –20ºC till further use.

Isolation of RNA and synthesis of cDNA: Total RNA was isolated from *A. caninum* using Trizol reagent following the manufacturer's (Gibco BRL) recommendations. The isolated total RNA was used to prepare cDNA by reverse transcription using oligo dT primer following the standard protocol described in AccuScript high fidility first strand cDNA synthesis kit (Stratagene, USA).

Cloning, expression and purification of AcCP2 protein: Oligonucleotide primers, i.e. AcCP2-F containing *Eco*RI restriction enzyme site (5'-CCAGGAATTCATACTCG CTGCATTATTAGTAACAG-3') and AcCP2-R containing *Xho*I restriction enzyme site (52 -ACGTCTCGAGCA CTCTCATCACTCCACTGACC -3) were designed using published sequence from GenBank (U18912) and used for amplification of 1023 bp gene of AcCP2; encoding 38.6 kDa AcCP2 protein. The AcCP2 gene was PCR amplified from cDNA in a 25 µl reaction mixture containing 0.25 µl of Dream *Taq* polymerase (10 U/µl), 10 pmol of each primer and 0.2 mM concentration of each dNTPs for 30 cycles (94°C for 60 sec, 60°C for 90 sec and 72°C for 60 sec).

The PCR product was gel purified using MinElute® Gel Extraction kit (Qiagen, GmbH, Germany), digested with *Eco*RI and *Xho*I restriction enzymes, and cloned into pET-32a(+) and expressed as thioredoxine (Trx) fusion protein in BL21 (DE3). Recombinant protein expression was then induced with 1 mM IPTG.

Polyhistidine tagged fusion protein (rAcCP2) was purified under denaturating conditions using 8M urea. The protein was eluted with elution buffer containing ascending grades of imidazole (10 mM, 40 mM, 80 mM, 100 mM and 200 mM) in 8 M urea in equilibration buffer and analyzed by SDS-PAGE. Finally the recombinant protein was renatured by dialysis against decreasing molar concentrations of urea and finally in PBS (pH 7.4). The renatured purified protein was quantified by Bradford protein assay kit (Amresco) following manufacturer's instruction and kept at –20°C till further use.

Western blot analysis: For confirmation that the recombinant His-tag fusion protein was expressed from the desired gene, the recombinant AcCP2 protein was checked by Western blotting using 1: 2000 dilution of Ni-NTA HRP conjugate (Qiagen, USA). Further, the specific immunoreactivity of rAcCP2 was assessed by western blot analysis using known *A. caninum* positive and negative dog sera (1: 20 dilution) and subsequently probed with goat anti-dog IgG HRP conjugate (Bethyl, USA) at 1: 2500 dilution in PBS.

The cross-reactivity of recombinant AcCP2 with serum samples from dogs infected with *Toxocara canis*, *Dirofilaria immitis*, *Babesia canis vogeli*, *Babesia gibsoni*, *Ehrlichia canis* and *Hepatozoon canis* was evaluated by western blot analysis as above.

Indirect ELISA: An indirect-ELISA with rAcCP2 was standardized for detection of specific antibodies to *A. caninum* in dogs. For standardization of indirect ELISA, 20 *A. caninum* positive dog sera and 15 negative sera samples from healthy dogs reared in disease free shed of Animal Nutrition Division, Indian Veterinary Research Institute, having no history and clinical signs of hookworm infection were used. The chequer board titrations were carried out to optimize the concentrations or dilutions of antigen (0.25 µg/well), serum samples (1: 200 dilution), secondary antibody (1: 30000 dilution) and substrate solution in this assay.

The individual wells of a 96-well microtiter plate (Nunc, Maxisorp, Denmark) were coated with 100 µl of purified recombinant AcCP2 protein (2.5 µg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The plate was incubated at 4ºC overnight. Excess unbound antigen was removed from the wells by washing three times with PBS-Tween buffer. The free binding sites in each well of microtiter plate were blocked for 1 h at 37ºC with 200 µl of 5% skimmed milk powder prepared in PBS (pH 7.4). Following washing, 100 µl of serum samples diluted 1: 200 with PBS were applied to the individual wells in duplicate and incubated for 1 h at 37ºC. After washing, 100 µl of goat anti-dog HRP-conjugate secondary antibodies (Bethyl, USA) at 1:30000 dilution in PBS (pH 7.4) was added to each well and incubated further for 1 h at 37ºC. After final wash for 3 times with PBS-T, 100 µl of O-phenylenediamine dihydrochloride (OPD) substrate (Amresco) dissolved in citrate-phosphate buffer (pH 5.0) with 30% H_2O_2 was added to individual wells. The reaction was allowed to develop for 15 min in dark and subsequently stopped by adding 100 µl of 1M H_2SO_4 to each well. Absorbance at 492 nm was measured by using microplate reader (SPECTRA MAX M5, USA).The cut-off value of the assay was determined using the mean value of optical density (OD) plus 3 standard deviations (SD) of the OD values of 15 *A. caninum* negative dog sera. The ODs above the cut off value were treated as positive.

The sensitivity and specificity of the assay was determined by using sera samples from 123 dogs, out of which 48 were detected positive for *A. caninum* eggs by microscopic examination of faeces, while the remaining 75 were faecal negative. Besides this, serum samples from dogs monospecifically infected with *Toxocara canis* (3), *Dirofilaria immitis* (1), *Babesia canis vogeli* (6), *Babesia gibsoni* (6), *Ehrlichia canis* (7) and *Hepatozoon canis* (2) were used to check the cross-reactivity of AcCP2 protein of *A. caninum* with these common parasites of dogs.

Sensitivity and specificity were calculated as, sensitivity = (TP/TP + FN) × 100 and specificity = (TN/TN + FP) × 100; where, TP indicates true positive (samples

positive in both microscopy and ELISA); FN indicates false negative (samples positive in microscopy but negative in ELISA); TN indicates true negative (samples negative in both microscopy and ELISA); FP indicates false positive (samples negative in microscopy but positive in ELISA).

Statistical analysis: Statistical analysis for determination of significant differences ($P<0.05$) between OD values of known *A. caninum* positive and negative dog sera in ELISA was done using Student's *t* test in GraphPad Prism version 4.0 and SPSS version 16.0 (SPSS, Inc., Chicago, USA). The test results were considered significantly different with P value < 0.05.

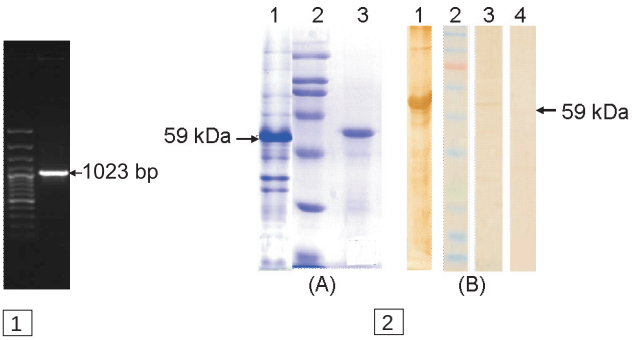
RESULTS AND DISCUSSION

Cysteine proteinase genes, encoding cathepsins B and/or L, have been reported in many nematodes viz. *Ancylostoma caninum* (Harrop *et al.* 1995a, b, Yang *et al.* 2011), *Haemonchus contortus* (Pratt *et al*. 1990), *Strongylus vulgaris* (Caffrey and Ryan 1994), *Toxocara canis* (Loukas *et al*. 1998), filarial nematodes (Guiliano *et al*. 2004) and *Caenorhabditis elegans* (Ray *et al*. 1992), and in the trematodes viz. *Fasciola hepatica* (Heussler and Dobbelaere 1994), *F. gigantica* (Grams *et al.* 2001) and *Schistosoma mansoni* (Smith *et al.* 1994). Since these proteases have a major role in various physiological processes and are expressed in the early developmental stages of the parasite, they may prove useful in the development of vaccines, anthelminthic drugs and immunodiagnostics for use in canines as well as in humans. Native or recombinant cysteine proteases have been used for the diagnosis of schistosomosis (El-Sayed *et al*. 1998), fasciolosis (Varghese *et al*. 2012), clonorchiosis (Nagano *et al*. 2004) and sparganosis (Rahman *et al.* 2014).

The present work is an attempt to serologically diagnose hookworm infection in dogs by indirect ELISA using recombinant AcCP2, a Cathepsin B protease of *A. caninum*. Since, AcCP2 is secreted by both the adult and larval hookworms (Yang *et al.* 2011), it may prove to be a useful candidate for use in serodiagnosis of hookworm infections. In the present study, 1023 bp AcCP2 gene was amplified (Fig. 1), cloned and sequenced for decoding the genetic information. The nucleotide sequence analysis of AcCP2 gene of Bareilly (India) isolate showed 98% identity with AcCP2 of Australian isolate (U18912), 89% with AcCP1 of Australian isolate of *A. caninum* (U18911), 87% with *A. duodenale* from China (JQ762435) and 65% with *H. contortus* cysteine proteinase (HMCP4) from Bareilly (GQ223787). The nucleotide sequence of AcCP2 gene was submitted to GenBank (KJ776604).

The AcCP2 gene was cloned into pET-32a(+) and a high level expression of rAcCP2 protein of 59 kDa as a fusion protein with Trx at 8 h post-IPTG induction was noticed on SDS-PAGE analysis. This polyhistidine tagged fusion protein (rAcCP2) was purified under denaturating conditions using 8 M urea (Fig. 2A). On western blotting, the recombinant fusion protein showed specific immunoreactivity with *A. caninum* infected dog serum at approximately 59 kDa but not with uninfected serum (Fig. 2B). Western blot analysis of the rAcCP2 protein did not show any cross-reactivity with serum samples from dogs infected with *Toxocara canis, Dirofilaria immitis, Babesia canis vogeli, Babesia gibsoni, Ehrlichia canis* and *Hepatozoon canis*.

For evaluation of rAcCP2 as serodiagnostic reagent for *A. caninum* infection, a panel of sera from dogs infected with *A. caninum*, uninfected sera and sera from dogs infected with other common parasitic infections were used. The cut off value for negative sera in indirect ELISA was 0.306 as determined by mean value (0.159) plus three standard deviations (3 × 0.051) of the OD values obtained from 15 known negative dog sera. The standardized assay clearly differentiated between *A. caninum* infected positive and uninfected negative dog sera, except in one case (out of twenty) where a positive sera was diagnosed as negative with an O.D. value of 0.228 (Fig. 3). No cross reactivity with other common parasites of dog viz. *Toxocara canis, Dirofilaria immitis, Babesia canis vogeli, Babesia gibsoni, Ehrlichia canis* and *Hepatozoon canis* was noticed in the standardized ELISA. Previous studies conducted by Loukas



Figs 1–2. 1. PCR ampilification of rAcCP2 gene. 2. Characterization of recombinant AcCP2 protein. (A) SDS-PAGE analysis of purified rAcCP2 fusion protein (Lane 1, unpurified rAcCP2; Lane 2, Molecular weight marker; Lane 3, purified rAcCP2). (B) Western blot analysis (Lane 1, rAcCP2 probed with Ni-NTA conjugate; Lane 2, molecular weight marker; Lane 3, rAcCP2 probed with dog sera naturally infected with *A. caninum*; Lane 4, negative control sera).

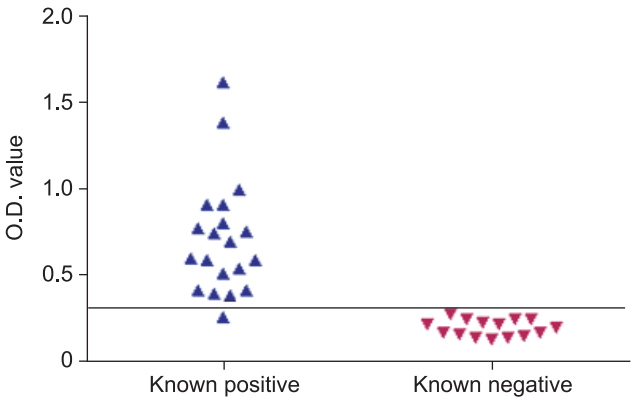


Fig. 3. rAcCP2 protein based indirect ELISA using known negative (15) and known positive (20) dog serum samples (Cut-off value of 0.306 is indicated by a line)

et al. (1992) reported that excretory-secretory (ES) products of *A. caninum* were more discriminating than somatic antigens in the ELISA and the IgG/ES-ELISA was specific with occasional cross-reactions.

Out of the 48 *A. caninum* faecal positive sera from dogs, 46 (95.8%) were tested positive (OD>0.306) by indirect ELISA. However, 14 (18.7%) out of 75 faecal negative samples were also tested positive (OD>0.306) by indirect ELISA. This may be due to early stage of infection (prepatency) or due to presence of inhibited larvae of *A. caninum* in these dogs or due to persistence of antibodies even after elimination of the parasites from the host. It has been reported that increased IgG antibody levels are recorded in hookworm infected dogs at 2–4 weeks post-infection (Dias *et al*. 2013). Significant differences ($P<0.05$) in mean OD values between ELISA positive dog sera (0.691±0.077) and ELISA negative dog sera (0.159±0.012) were noticed by Student's *t* test. Based on the results, the sensitivity and specificity of rAcCP2 based indirect ELISA was calculated to be 95.8% and 84.3%, respectively, when microscopic examination of faeces was taken as a reference test.

The results of the present study indicate that indirect ELISA with recombinant AcCP2 protein might be appropriate in a large scale epidemiological screening and serological diagnosis of *A. caninum* infection. However, extensive studies are required to further refine the assay and to determine the diagnostic sensitivity and specificity of indirect ELISA using recombinant AcCP2 protein.

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