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Short communication

Development of cathepsin-L cysteine proteinase based Dot-enzyme-linked immunosorbent assay for the diagnosis of *Fasciola gigantica* infection in buffaloes

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ABSTRACT

Native cathepsin-L cysteine proteinase (28 kDa) was purified from the excretory secretory products of *Fasciola gigantica* and was used for sero-diagnosis of *F. gigantica* infection in buffaloes by Dot-enzyme-linked immunosorbent assay (Dot-ELISA). The test detected *F. gigantica* field infection in these animals with a sensitivity of ~90%. No specific IgG antibody binding was displayed by sera obtained from 76 buffaloes considered to be *Fasciola* and other parasite-free by microscopic examination of faeces and necropsy examination of liver, rumen and intestine. Additionally, sera from 156 *Fasciola*-free buffaloes, yet infected with *Gigantocotyle explanatum*, *Paramphistomum epiclitum*, *Gastrothylax* spp., *Strongyloides papillosus* and hydatid cyst were all negative, indicating that *F. gigantica* cathepsin-L cysteine proteinase does not cross-react with these helminth parasites in natural infection of the host. The data indicated that cathepsin-L cysteine proteinase based Dot-ELISA reached ~90% sensitivity and 100% specificity with relation to above parasites in the detection of bubaline fasciolosis. The present Dot-ELISA diagnostic assay is relevant to the field diagnosis of *F. gigantica* infection in buffaloes.

1. Introduction

Tropical fasciolosis caused by *Fasciola gigantica* is a relentless constraint on the growth and productivity of cattle, buffaloes and sheep in the tropical countries (Spithill et al., 1997; Mehra et al., 1999; Yamasaki et al., 2002). Early prepatent diagnosis of fasciolosis in domestic ruminants is crucial for arresting its negative impact on productivity and sustained efforts towards developing specific serodiagnostic tests for early detection of fasciolosis in animals have shown a significantly high level of sensitivity with several antigenic preparations (Guobadia and Fagbemi, 1997; O'Neill et al., 1998; Cornelissen et al., 2001; Dixit et al., 2002, 2004; Yadav et al., 2005; Raina et al., 2006; Estuningsih et al., 2009; Anuracpreeda et al., 2011). *Fasciola* worms release substantial amounts of excretory–secretory products (ESPs) and cysteine proteinases are predominant antigens present in these *ex vivo* ESPs (Dalton and Heffernan, 1989; Smith et al., 1993; Jefferies et al., 2001; Morphew et al., 2007). The excretory–secretory products produced by *Fasciola* are key players in understanding the host–parasite interaction and offer targets for diagnosis, chemo- and immunotherapy (Morphew et al., 2007). Cathepsin-L cysteine proteinases have been exploited in the immunodiagnosis of *Fasciola* infection in human and animals and have given encouraging results for sensitive and specific diagnosis of the disease. The 28 and 26–27 kDa molecules of the excretory and secretory products were shown to essentially consist of cathepsin-L cysteine proteinases (Dixit et al., 2002, 2004) and when 28-kDa cysteine

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proteinase was assayed in the prepatent diagnosis of fasciolosis, it detected *F. gigantica* experimental infection in bovine, bubaline and ovine hosts with 100% sensitivity (Dixit et al., 2002, 2004; Sriveny et al., 2006). This antigen was used in the ELISA format for the detection of bubaline fasciolosis and detected the infection with high sensitivity and specificity under field conditions (Raina et al., 2006). Present study aimed at developing a cysteine proteinase based Dot-ELISA in the immunodiagnosis of bubaline fasciolosis for field conditions.

2. Materials and methods

2.1. Parasite collection

Fasciola gigantica flukes collected in physiological saline from the liver of buffaloes, slaughtered at a local abattoir, Bareilly, U.P., were brought to the laboratory and washed to remove host-origin material with normal saline.

2.2. Purification of cathepsin-L cysteine proteinase

Cathepsin-L cysteine proteinases were purified from the *F. gigantica* regurgitant released *in vitro* in the excretory secretory products. Briefly, viable flukes were incubated in RPMI-1640 medium, pH 7.2 (Biological Industries, Israel) with one fluke/ml of the medium, supplemented with 0.2% sodium bicarbonate, 30 mM HEPES and 25 mg/l gentamycin. After 3–4 h incubation at 37 °C, the culture medium with *in vitro* released parasite regurgitant, was centrifuged at 10,000 × *g* for 20 min and supernatant used for precipitation of the cysteine proteinase. Chilled ethanol was added to the culture supernatant, drop by drop, to a final concentration of 60% (v/v) and the suspension incubated at −20 °C overnight. Proteins precipitating at 60% (v/v) ethanol concentration were pelleted at 6000 × *g* (20 min, 4 °C) and discarded. Ethanol concentration in the supernatant was subsequently raised to 75% (v/v) and supernatant incubated overnight at −20 °C. The precipitated proteins were centrifuged at 6000 × *g* (20 min, 4 °C). The cysteine proteinases thus precipitated at 75% (v/v) ethanol concentration were rinsed in 70% ethanol, air dried and resuspended in PBS, pH 7.2.

2.3. Ion-exchange chromatography

Three grams of DEAE Sephadex-A25 (Sigma Chemical Company, USA) were swollen in distilled water for 3–4 h and the resin was suspended in 10 bed volumes of 0.1 M NaOH. The resin was washed thoroughly with distilled water until its pH became neutral, re-suspended in 0.1 M HCl and washed as above. Thereafter, a 10 ml column was packed with the resin and equilibrated with 10–20 bed volumes of 100 mM, 50 mM, 20 mM and 10 mM sodium phosphate buffer (pH 8.0), respectively. Cathepsin-L cysteine proteinase earlier dialysed in 10 mM phosphate buffer (pH 8.0) was loaded onto the pre equilibrated column. Ion-exchanger bound cathepsin-L cysteine proteinase was eluted with a sodium chloride, non-linear gradient (0.1–0.5 M) in 10 mM phosphate buffer (pH 8.0). Purified protein was resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and characterized as cathepsin-L cysteine proteinase (Yadav et al., 2005).

2.4. Dot-ELISA

Dot-ELISA with soluble cathepsin-L cysteine proteinase was carried out with the antigen concentrations of 1 μg, 500 ng, 200 ng and 100 ng, respectively, blotted to the nitrocellulose membrane (Biorad, California, USA). The antigen was reconstituted in PBS (pH 7.2) and dots of 1 μl volume of the antigen were applied to the nitrocellulose strips. The antigen spots were air dried, blocked with 5% skimmed milk and washed with PBS-Tween-20 (wash buffer). Sera from *F. gigantica* infected buffaloes collected from a local abattoir and other areas were screened for anti-*Fasciola* antibodies at serial dilutions of 1:50–1:200. Sera collected from buffaloes infected with helminths other than *Fasciola* were also probed in the immunoassay and immuno-reactivity of the sera was probed with rabbit anti-bovine IgG–horse radish peroxidase (HRP) conjugate (Sigma Chemical Company, USA) at 1:2000 dilution. ELISA dots were developed with diaminobenzedine substrate (Sigma Chemical Company, USA) (DAB, 8 mg; PBS pH 7.2,10 ml; 8% nickle chloride, 50 μl; hydrogen peroxide, 10 μl, for a total 10 ml substrate volume). Sera positive for *F. gigantica* infection showed purple dots on the membrane while negative sera showed no dots.

2.5. Sensitivity and specificity

Sera from buffaloes from *F. gigantica* non-endemic regions, mono-infected with helminths other than *F. gigantica*, were used to determine specificity of Dot-ELISA. The sensitivity was determined in *F. gigantica* infected buffaloes from *F. gigantica* endemic areas. The sensitivity ands specificity were calculated as, sensitivity = ELISA positive × 100/true positive and specificity = ELISA negative × 100/true negative.

3. Results

Cathepsin-L cysteine proteinase (28 kDa) was purified to complete homogeneity following above purification protocol and the optimum concentration of the antigen for membrane coating was standardized to 200 ng/dot using chequer board titration. The results of the Dot-ELISA were read on the basis of colour development on nitrocellulose membrane, with positive reactions being read as purple dots and negative results with no dots (Fig. 1). Sensitivity of the Dot-ELISA was checked with the field sera collected from buffaloes at slaughter from *F. gigantica* endemic region of India. Fluke burden in the host was determined on necropsy as heavy (50 flukes and higher), moderate (11–50) and low (1–10). Out of 136 buffaloes, positive for *F.gigantica* infection by liver and faecal examination, 122 animals were positive by Dot-ELISA. The sera of the remaining 14 animals that were positive for adult flukes in the liver did not react with the antigen. The animals screened in this assay harboured either *F. gigantica* mono-infection or a mixed infection of *F. gigantica* with other helminths like