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Research Article

28 kDa *FASCIOLA GIGANTICA* CYSTEINE PROTEINASE (FgCL3) BASED SERO-DIAGNOSIS OF BOVINE TROPICAL FASCIOLOSIS IN THE DAIRY ANIMALS OF PUNJAB, INDIA

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ABSTRACT: Tropical fasciolosis is one of the neglected diseases affecting a large proportion of dairy animals in developing countries like India and its economic assessment has revealed huge production losses in affected animals. Reliability of the traditional diagnostic methods lacking sensitivity in prepatent and chronic cases stipulates early and accurate diagnosis. Serodiagnosis is a fast and reliable diagnostic approach for fasciolosis and improved sensitivity, specificity, and accuracy have been obtained with the purification and use of cysteine proteinases as antigens even in naturally infected animals. The present study has been designed to standardize the cysteine proteinase-based ELISA for the assessment of serological prevalence of tropical fasciolosis in dairy animals of Punjab, India. The study highlights the use of cysteine proteinase over coprological approaches for early detection of fasciolosis in dairy animals to minimize the production losses in dairy animals of the state.

Keywords: Cysteine proteinase, Dairy animals, ELISA, Punjab, Tropical fasciolosis.

INTRODUCTION

Fasciolosis, commonly called liver fluke disease, is of paramount importance worldwide due to the wide spectrum of definitive hosts. *Fasciola gigantica*, mostly prevalent in Asia and Africa, is a major cause of production losses in ruminants including dairy animals. Losses associated with the disease include reduced milk yield, poor carcass quality, liver condemnation, and reproductive failure. The annual worldwide losses in animal productivity due to fasciolosis have been estimated at over US$ 3 billion [1]. Reports of the infection in more than 17 million people worldwide and its significant public health concerns are also documented [2]. Microscopic fecal examination is the conventional gold standard diagnostic approach with limited utility in early as well as chronic infections. Liver injury is indicated by early biochemical changes [3]. In the infected animals, however, the role of fasciolosis still needs laboratory confirmation stipulating a simple, sensitive, and specific test for early diagnosis of fasciolosis. In this regard, serodiagnosis has yielded encouraging findings earlier [4, 5, 6]. Especially, with the purification of cysteine protease antigen that can yield improved accurate and early diagnosis of fasciolosis [7, 8]. Several reports of cysteine proteinase-based *F. gigantica* diagnosis are available across the country [3, 10, 11] with no cross-reactivity [12, 13]. So, the present study was planned to develop a specific immunodiagnostic test for fasciolosis in naturally infected dairy animals of Punjab, India.

MATERIALS AND METHODS

Collection of parasites

Live flukes were collected from infected livers of buffaloes slaughtered at large animal slaughterhouses of Punjab, *viz.* Ludhiana, and Chandigarh. Infected liver was brought as such in the Department of Veterinary Parasitology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. Viable and intact flukes were collected from the liver and kept in lukewarm

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phosphate-buffered saline (PBS) while damaged flukes were discarded.

Collection of samples

Simultaneous fecal and blood samples (5mL) from a total of 214 animals comprising 118 buffaloes and 96 cattle were collected from slaughterhouses and Veterinary hospitals respectively located at Ludhiana and Chandigarh as well as from different districts of five agro-climatic zones. Blood was collected in sterile tubes without anticoagulant, and allowed to clot at room temperature in an inclined position, serum was separated and transferred to microcentrifuge tubes. These tubes were transported in ice-packed conditions to the laboratory, centrifuged at 2500 rpm for 10 min, and clear sera and stored at -20ºC in a deep freezer.

Coprological examination

The fecal samples were first subjected to standard qualitative examination using the direct smear method and concentration techniques (sedimentation) for detection of the *Fasciola* sp. eggs. The eggs were identified based on the morphological features as described by Soulsby [14].

Purification of cysteine protease

The purification of the 28 kDa cysteine proteinase from adult *F. gigantica* regurgitant was achieved following the earlier used protocol [15] with some modifications. Scrupulously washed viable flukes were incubated at 37ºC (1 fluke/mL) in RPMI-1640 media (pH 7.3), containing 2% glucose, 30 mM Hepes, and 40 mg/L gentamicin. After 3h of incubation, the culture medium containing *in vitro* released regurgitant was centrifuged (5500×g, 4ºC, 1h) and was frozen (at -20ºC) until required. After thawing the regurgitant, cold ethanol was added dropwise into the chilled regurgitant, until final ethanol concentration of 60% (v/v) was achieved. The mixture was equilibrated at – 20ºC for 18 h and thereafter pelleted at 6300 × g (40 min, 4ºC). The pellet was discarded and the supernatant was taken to a final concentration of 75% (v/v) ethanol, equilibrated overnight at -20ºC, and then centrifuged (6300 × g, 40 min, 4ºC). The pellet obtained from 75% ethanol precipitation, was washed in 100% ethanol, absolutely dehydrated, and resuspended in distilled water.

Protein estimation

Protein estimation of the antigen was done by Nano-Drop Spectrophotometer (Nano-Drop Technology USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The purity of the protein was checked on SDS-PAGE (12%) under reducing conditions [16]. Protein was electrophoresed at 80V through stacking gel and 120V through separating until tracking gel reached the bottom. The gel was stained with 0.15% Coomassie stain and destaining was done using 7.5% acetic acid.

Western blot analysis

Immunoblotting was performed as per standard [17] with slight modifications. The protein separated in the gel by SDS-PAGE were transferred onto pre-stained protein molecular weight standards (MBI Fermentas, 150-10kDa) nitrocellulose membranes (NCM) in transfer buffer (0.025M Tris 0.192M glycine, pH 8.3 with 20% v/v methanol) at a constant current of 40 mA for two hours using semidry transfer apparatus (Atto, Japan).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA was performed as per earlier studies [10,13] with slight modifications using microtiter plates (Nunc, Denmark). Blocking was done with 5% skimmed milk powder in PBS/Tween (SMP/PBS/T) for 1 h at 37ºC. Rabbit anti-bovine HRPO conjugate (Genei, Bangalore) and ortho-phenylene diamine (OPD, Sigma) at a concentration of 1 mg/mL in a sodium citrate buffer, pH 4.6 containing 30% hydrogen peroxide (10 µL) were used for color development and the reaction was stopped by 2M H_2SO_4. The absorbance was read at 490 nm on a microplate reader. The results, expressed as the mean of the optical density (OD) were obtained from duplicate samples. The cut-off value in the ELISA was determined as the average optical density value of six negative controls plus three standard deviations using 6 known positive and 6 known negative controls. After standardization, indirect ELISA was carried out in 214 serum samples of 118 buffaloes and 96 cattle from Punjab and was compared with their coprological examination results.

RESULTS AND DISCUSSION

Coprological results

Out of 214 samples examined, 13 (6.07%) samples were found positive for fasciolosis. The animal-wise analysis presented a statistically non-significant higher prevalence rate of fasciolosis (Fig. 1) in the buffalo population (6.78%) as compared to the cattle population (5.21%) of the region with complete details in Table 1.

Table 1. Prevalence (%) of bovine fasciolosis by coprological and serological examination.

Animals	Examined	Coprological examination	Serological examination	x^2 (p value)
Cattle	96	5 (5.21%)	12 (12.50%)	3.1624 (0.0753)
Buffalo	118	8 (6.78%)	20(16.95%)	5.835* (0.0157)
Total	214	13 (6.07)	32 (14.95%)	8.967** (0.0045)
χ^2 (p value)	--	0.2291(0.891)	0.823 (0.662)	--

[**Highly significant (p<0.01); *Significant (p<0.05)].

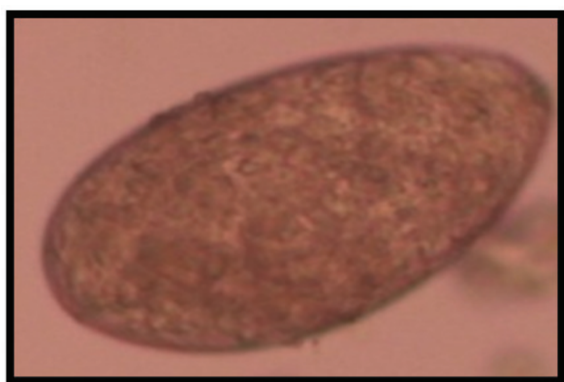

Fig. 1. *Fasciola* sp. Egg.

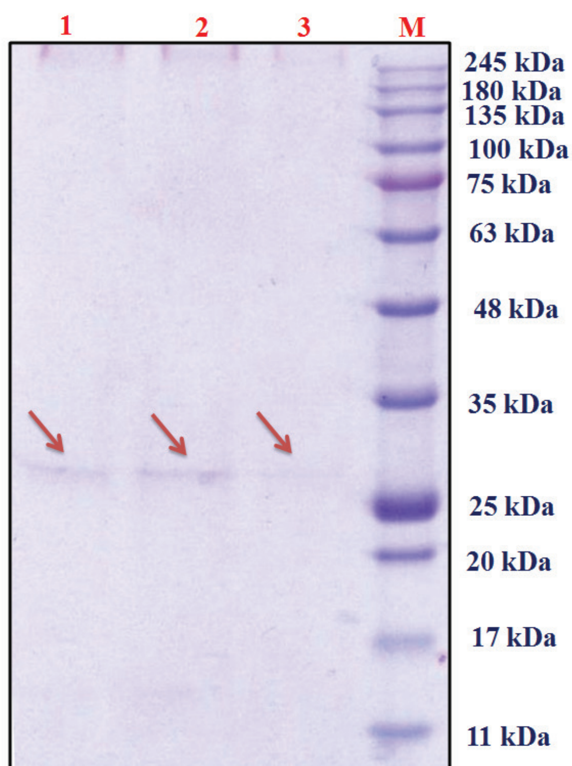

Fig. 2. SDS-PAGE showing bands of purified 28 kDa cysteine protease (Lane M = Marker; Lane 1, 2, 3 = Cysteine protease).

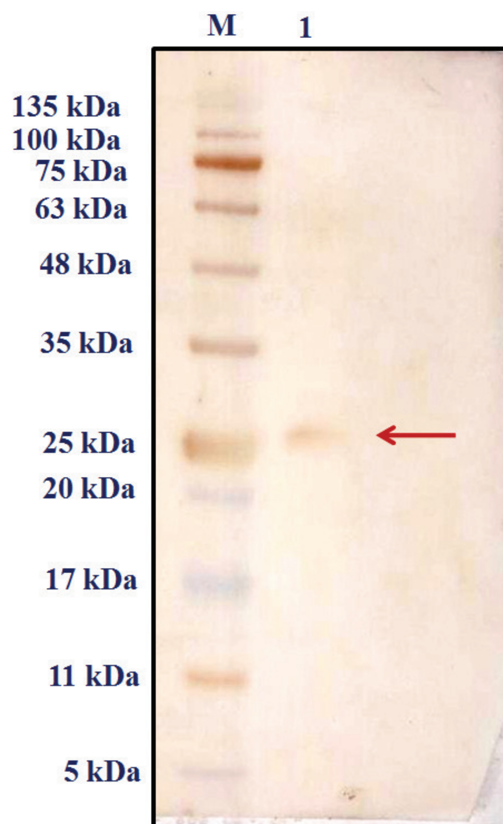

Fig. 3. Western Blot showing strong reaction of 28 kDa cysteine protease with the sera of known positive animal (Lane M = Marker; Lane 1 = Cysteine protease).

The coprological examination has been believed to be the golden standard method for the diagnosis of fasciolosis for ages with a far-fetched solution for chronic as well as immature infections. Fasciolosis has been recorded as endemic in the buffalo population [18] and cattle population [19] from different parts of the state and availability of the favorable factors was presented. The coprological prevalence rate of fasciolosis reported in the present study is higher than the findings of earlier studies in the region [20,21] while in agreement with the reports from other parts of the country [22,23]. Variation in prevalence rates may be due to the difference in the geographical conditions and topography of the study areas [23].

Serological results

Purification of cysteine protease

A total of 200 intact and viable flukes were recovered from the infected livers yielding 18.72 mg cysteine proteinase with an average yield of 93.6 µg per fluke. The concentration of the final dilution was 1.93 mg/ml. SDS-PAGE resulted in purified 28kDa cysteine protease (Fig. 2). The 28 kDa cysteine proteases are abundantly synthesized in adult flukes (0.5-1.0 mg/adult fluke/h) with a swift secretion (1 hr approx) into the gut lumen [24]. Cysteine protease yield per fluke is in agreement with the earlier findings [3].

Optimization of Western blot and ELISA

Antibody detection has shown potential as a preferable alternative with the condition of high specificity of antigen [3,9,13]. Standardization analysis revealed 2µg/mL for cysteine protease 1:100 dilution of serum and 1:1000 dilution of anti-bovine HRPO conjugate as optimum dilutions for western blot analysis and developed a strong reaction of 28 kDa cysteine protease Cathepsin-L with the sera of known positive indicating its immunodominant characteristic (Fig. 3). Similarly, optimum dilutions of cysteine protease, test serum, and anti-bovine HRPO conjugate were identified as 2µg/ml, 1:100, and 1:5000 respectively were identified by the checkerboard analysis for ELISA conduction. The cut-off value in indirect ELISA was 0.152 whereas, the average optical density value for the positive and negative control values were 0.261 and 0.101 respectively.

Serological prevalence and comparative analysis

The serological investigation presented a highly significant ($p<0.01$) increase in the overall prevalence rate (14.95%) of fasciolosis. Also, a significantly higher ($p<0.05$) increase in the prevalence rate was reported in the buffalo population (16.95%) of the region. However, variation in terms of the higher prevalence rate of cattle population (12.50%) as compared to the coprological prevalence was statistically nonsignificant with complete details in Table 1. Several reports of high sensitivity, specificity, and accuracy of cysteine protease are available [3, 7, 8]. However, sensitivity has shown a slight reduction to 97% in the case of natural infections [13]. Previously, several researchers had employed 28 kDa cysteine protease as an antigen for the diagnosis of bovine fasciolosis [3, 11, 13]. The absence of any cross-reaction to the natural infections of *Schistosoma indicum*, *Schistosoma spindale*, *P. epiclitum*, *Gastrothylax* sp., *Gigantocotyle explanatum*, Hydatid and *Strongyloides papillosus* in the bubaline host has confirmed the antigen specificity of 28 kDa cysteine protease [13]. Furthermore, it is unknown if the fluke burden will affect the antibody titer relative to the test's sensitivity, which depends on each host's antibody response [13]. To the best of our knowledge, the current study is the first to evaluate the serological prevalence rate of bovine fasciolosis in the dairy animals of Punjab, India, and to standardize the cysteine protease-based immunodiagnostic test. This work further emphasizes the potential of 28 kDa cysteine protease as an antigen for bovine fasciolosis immunodiagnosis based on cysteine protease, which could lead to the efficient management of the illness in the area.

CONCLUSION

The current study found that Punjab's dairy cows had a significantly higher statistically significant prevalence of bovine fasciolosis than coprologically reported. The use of advanced techniques for bovine fasciolosis detection is highlighted in the current study. The study also demonstrates that the 28 kDa cysteine protease is a suitable antigen for the serological identification of bovine animals in animals that are naturally infected. Additionally, a serological evaluation of the prevalence of bovine fasciolosis in dairy animals in Punjab using the 28 kDA cysteine protease assay could be immensely helpful in developing efficient disease control strategies for the region.

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