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Identification of immunodominant fraction of *Paramphistomum epiclitum* and its evaluation for use in the serodiagnosis of paramphistomosis by ELISA

SHAFIYA IMTIAZ RAFIQI[1], SAKEER HUSSAIN K M[2], HIRA RAM[3], RAJAT GARG[4], SAROJ KUMAR[5], M K SINGH[6] and P S BANERJEE[7]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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ABSTRACT

The present study was undertaken to identify and purify the immunodominant fractions from the excretory secretory (ES) antigen of *Paramphistomum epiclitum*, a predominant amphistome species infecting ruminants in India. ES antigen was prepared and characterized using SDS-PAGE and Western blot analysis. Major polypeptides of molecular weight 11, 22, 28, 31, 33, 39, 52, 59, 63 and 72 kDa were visualized in SDS-PAGE. Polypeptides (9) of 11, 14, 16, 22, 31, 33, 39, 63 and 72 kDa showed immunoreactivity in Western blot analysis. The whole ES antigen of *P. epiclitum* was initially concentrated using PEG-8000 followed by spin-X UF concentrator with 10 kDa cutoff range and subsequently fractionated by size exclusion chromatography using Sephadex G-25. Cross reactivity of the *P. epiclitum* ES antigen was studied with positive sera of *F. gigantica* and *H. contortus*. Based on the cross reactivity profile, the low molecular weight antigenic fraction with 11 kDa polypeptide was selected for further use in indirect-ELISA. Bovine serum samples (258) were tested with optimized ELISA. Sensitivity of the ELISA was calculated as 75.0%, while the specificity was 85.0%. The percent positive and negative predictive values for the test were 70.78 and 87.57%, respectively.

Key words: ES antigen, Immunodiagnosis, Low molecular weight protein, Paramphistomosis

Immature paramphistomosis is a neglected trematodal disease of ruminants (Meshgi *et al*. 2009). About 70 species of amphistomes have been reported so far from different parts of the world, but only few are predominant in a particular agroclimatic region depending upon the presence of specific snail intermediate host required for completing the life cycle. In India, amphistomosis has a wide range of geographical distribution and ruminants are mostly affected by *Paramphistomum epiclitum* (Haque *et al.* 2011, Singh *et al*. 2012). Death rates due to this disease could be as high as 80–90% in domesticated ruminants in endemic belts (Khan *et al*. 2008). Acute disease caused by immature amphistomes results in poor digestion and inefficient nutrient absorption resulting in diarrhea, anorexia, anemia and weakness (Spence *et al*. 1996). Since the disease is caused by immature amphistomes migrating through intestinal mucosa, coproscopic analysis is futile and the condition remains undiagnosed due to lack of effective diagnostic tests. Tentative diagnosis based on history and clinical signs are not reliable as analogous symptoms can also be noticed in other diseases like paratuberculosis, liver fluke and roundworm infections as well as in copper deficiency (Radostits *et al*. 1994). Therefore, development of a serodiagnostic assay utilizing a suitable immunodiagnostic antigen of the most commonly occurring amphistome species is essentially required. Accordingly, the present study was undertaken to identify the immunodominant excretory secretory (ES) antigenic fraction of *P. epiclitum* and its further evaluation in ELISA for the diagnosis of infection in ruminants.

Present address: [1]Ph.D Scholar (shafiyaimtiaz@gmail.com), [3]Senior Scientist (hiraram.35@gmail.com), [4]Principal Scientist (rajatgarg_2000@yahoo.com), [7]Head (banerjeeparth62 @gmail.com), Division of Parasitology; [6]Scientist (drmithi07vet @gmail.com), Immunology Section. [2]Veterinary Officer (drsakeerhussain@gmail.com), Government of Kerela. [5]Research Associate (drsarojvet@gmail.com), ICAR-NRCE, Hisar.

MATERIALS AND METHODS

Collection and morphological identification of Paramphistomum epiclitum*:* Adult amphistomes were collected in PBS (pH 7.4) from the rumen of slaughtered buffaloes at a local abattoir (Bareilly, India) and transported to the laboratory, immediately. These flukes were washed three times in PBS (pH 7.4) and approx 150 *P. epiclitum* were sorted out after morphological identification (Dutt 1980).

Preparation of excretory-secretory (*ES*) *antigen:* Excretory-secretory (ES) antigen of *P. epiclitum* was prepared by incubating the adult flukes in PBS (pH 7.4) for 6 h at 37ºC in a sterile petridish (15 flukes in 20 ml of PBS). After 6 h, the parasites were removed and the

suspension was centrifuged at 12,000 rpm for 30 min at 4ºC. The supernatant was then filtered through syringe filter (0.45µm). ES product was concentrated initially using PEG-8000 and subsequently by spin-X UF concentrator (Corning, New York) with 10 kDa cut-off range. Concentration of protein in the ES antigen was determined using spectrophotometer (Nanodrop® ND 1000, USA). Cocktail protease inhibitor (Amresco, USA) was added in the antigen and stored at –20ºC after proper labeling.

Raising of hyperimmune sera: Hyperimmune serum was raised against the ES antigen of *P. epiclitum* in a adult male rabbit (New Zealand white) obtained from Laboratory Animal Resource Section of IVRI, Izatnagar as described by Harlow and Lane (1988). Animal experimentation was performed after getting the CPCSEA permission. Serum samples were tested by double immunodiffusion (DID) to ascertain optimum antibody titre against the ES antigen.

Characterization of whole ES antigen of P. epiclitum *by SDS-PAGE and Western blotting:* SDS-PAGE was carried out as per Laemmli (1970) for characterization and analysis of different polypeptides in the whole ES antigen of *P. epiclitum*. After electrophoresis, gel was stained with Coomassie brilliant blue G-250 and the molecular weight of different proteins were determined using gel documentation system (Syngene). Immunodominant polypeptides in the whole ES antigen of *P. epiclitum*, were identified by Western blot analysis using hyperimmune serum (1:500 dilution) raised in the study as well as serum samples (1:20 dilution) obtained from slaughtered buffaloes harbouring heavy infection of *P. epiclitum* at necropsy. Goat anti-rabbit IgG-HRP conjugate and sheep anti-bovine IgG-HRP conjugate were used as secondary antibodies at 1:3000 dilution. Immuno-reactivity of antigenic fractions was visualized after short incubation of membrane in diaminobenzidine (DAB) solution (Amresco, USA). Cross reactivity of the *P. epiclitum* ES antigen was evaluated using the serum raised against *Fasciola gigantica* and *Haemonchus contortus* by Western blot analysis.

Purification and characterization of immunogenic fraction: Gel filtration chromatography using Sephadex G-25 was performed for fractionation of antigenic molecules of *P. epiclitum* ES antigen and 30 fractions of 2 ml each were eluted with 0.15 M NaCl. Protein concentration of the individual fractions was measured using protein assay kit (Bradford method) and finally 3 fractions showing comparatively high OD values were selected and designated as fraction 1, 2 and 3 (F1, F2, and F3) based on size of bands in decreasing order. Different fractions were characterized by SDS-PAGE and Western blotting as described above. Low molecular antigenic fraction (F3) which did not show cross reactivity with *F. gigantica* and *H. contortus* was selected for its use in serodiagnosis.

Collection of positive and negative serum samples: Corresponding blood and fecal samples of slaughtered buffaloes were collected from the local abattoir of Bareilly (Uttar Pradesh, India) and transported to laboratory for screening and confirmation of amphistome positive and negative status of the samples. Sera samples (258: 84 from amphistome positive and 174 amphistome negative) were collected from slaughtered buffaloes. Serum was labeled and stored at –20ºC till further use. *F. gigantica* and *H. contortus* experimental serum raised in sheep were obtained from reference laboratories of the Parasitology Division.

Optimization of Indirect ELISA: An indirect-ELISA using the low molecular weight ES antigen fraction (F3) containing 11 kDa immunodominant antigen was standardized by checkerboard titration to evaluate its immunodiagnostic potential. The individual wells of a 96-well microtiter plate (Nunc, Maxisorp, Denmark) were coated with 50 µl of low molecular weight ES antigen of *P. epiclitum* (2 µg/ml) in 0.05 M carbonate bicarbonate buffer (pH 9.6). The plate was incubated at 4ºC overnight. The wells were washed thrice with PBS-Tween 20 (0.05% v/v), blocked with 5% skimmed milk prepared in PBS (pH 7.4) and incubated at 37ºC for 1 h. Wells of the plate were again washed three times with PBS-T and 50 µl of known positive (15) and negative serum samples (15) diluted in PBS (1:200) were added and incubated at 37ºC for 1 h. After washing the plate thrice, 50 µl of rabbit anti-bovine HRP-conjugate (Bethyl, USA) at 1:5000 dilution in PBS (pH 7.4) was added to each well and incubated at 37ºC for 1 h. Following washing, 50 µl of O-phenylene diamine dihydrochloride (OPD) substrate (Amresco, USA) dissolved in phosphate-citrate 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 later stopped by adding 50 µl of 1 M H_2SO_4 to each well. Absorbance at 492 nm was measured by using microplate reader (SPECTRA MAX M, Molecular devices, 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 known negative bovine serum samples. The OD values above the cutoff were treated as positive.

Screening of bovine sera samples by ELISA: Optimized ELISA was used for detection of antibodies against 11 kDa low mol wt. antigenic fraction of *P. epiclitum* in 258 bovine sera samples. Mean absorbance values of the duplicate samples were recorded.

Determination of sensitivity and specificity of indirect ELISA: Sensitivity and specificity of indirect ELISA was calculated using the formulae as described by Figueroa-Santiago *et al.* (2011). Percent positive and negative predictive value of the ELISA test was calculated using the formulae as described by Arjmand *et al*. (2014) for evaluation of Dot-ELISA for the diagnosis of *Fasciola* infection in cattle.

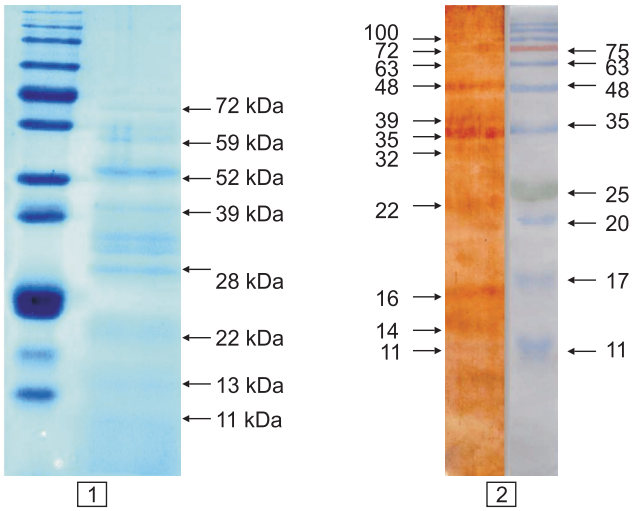
RESULTS AND DISCUSSION

In the present study, an attempt was made to characterize and identify the immunodominant antigenic fractions from the excretory secretory products of the *P. epiclitum*, which is one of the most predominant amphistome species infecting ruminants in India (Prasad and Varma 1999). SDS-PAGE analysis of the ES antigen revealed more than 10

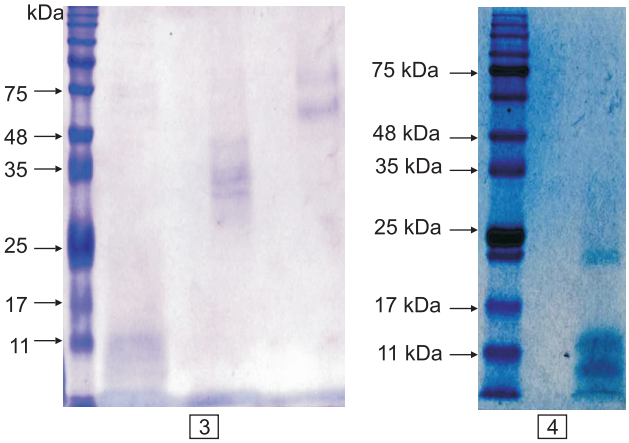
polypeptides in the range of 11 kDa to 72 kDa. Molecular weights of some major polypeptides resolved in polyacrylamide gel were approximately 11, 22, 28, 31, 33, 39, 52, 59, 63 and 72 kDa, along with few other bands of minor intensity (Fig. 1). In Western blot analysis of ES antigen, a total of 9 immunodominant polypeptides were recorded and their approximate molecular weights were 11, 14, 16, 22, 31, 33, 39, 63 and 72 kDa (Fig. 2). Saifullah *et al*. (2000a) also characterized the ES antigen of *Gastrothylax crumenifer* and recorded many polypeptides ranging from more than 29 kDa to less than 205 kDa. In the recent past, few studies have been performed to identify immunodiagnostic antigen(s) of amphistomes targeting the whole worm extract, somatic antigens or coproantigens (Saifullah *et al*. 2000b, 2013, Kaur *et al*. 2009, Arora *et al*. 2010, Tariq *et al*. 2011, 2013, Sakeer 2013, Shameem *et al*. 2016). However, the immunodominant antigens identified in the somatic antigen of amphistomes were found to be less specific when employed in immunodiagnostic assays (Kaur *et al*. 2009). The superiority of ES antigen in serodiagnosis of trematodal infections, viz. *Clonorchis sinensi*, *Paragonimus* sp., *F. hepatica*, *Opisthorchis felineus* and *Paramphistomum cervi* has been well documented (Choi *et al*. 2003, Narain *et al*. 2005, Awad *et al*. 2009, Gomez-Morales *et al*. 2013, Anuracpreeda *et al*. 2013). Saifullah *et al*. (2000a) also characterized the ES antigen of *Gastrothylax crumenifer* and recorded many polypeptides ranging from more than 29 kDa to less than 205 kDa.

Cross-reactivity is very frequently observed with crude antigens of helminths, which can be minimized by further purification of the diagnostic antigens (Fagbemi and Obarisiagbon 1991). Cross-reactivity of *P. epiclitum* whole ES antigen was evaluated using the sheep serum experimentally infected with *F. gigantica* and *H. contortus*. In western blot analysis, a total of 5 polypeptides of mol. wt. 28, 33, 59, 63 and 72 kDa showed immunoreactivity with *F. gigantica* positive serum, while no cross-reactive polypeptides were recognized with *H. contortus* positive serum.

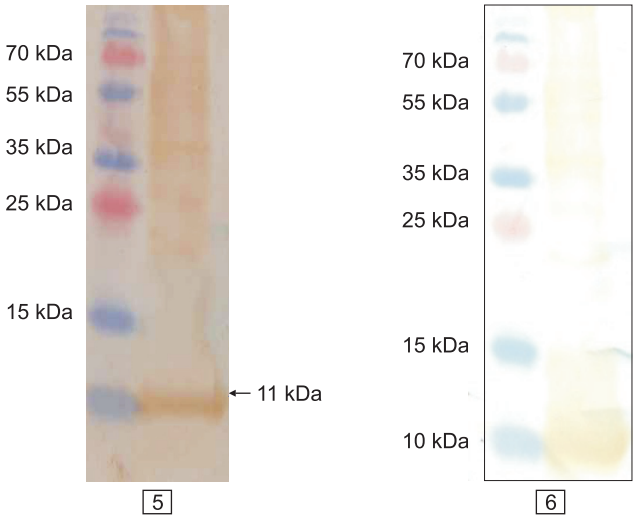
On size exclusion chromatography of ES antigen of *P. epiclitum*, peak OD values were recorded in fraction 6, 12 and 19 (designated as F1, F2 and F3). In fraction one (F1), polypeptides of approx. 72, 63 and 52 kDa were detected; while in fraction 2 (F2), polypeptides of 39, 33 and 31 kDa; and in fraction 3 (F3), polypeptides of 22, 13 and 11 kDa were noticed (Figs 3, 4). Western blot analysis of the selected fractions using the HIS raised in rabbit against the whole ES antigen of *P. epiclitum*, recorded 2 immunodominant antigenic band(s) in fraction one (F1) with approx. mol. wt. of 72 and 63 kDa. Similarly, immunodominant bands of 39 and 32kDa were recognized in eluted fraction 2 (F2), and a single immunodominant polpeptide of 11 kDa molecular weight showed immunoreactivity in the immunoblotting of fraction 3 (Fig. 5). Low molecular weight antigenic fraction (F3) showed immunoreactivity with cattle sera as well (Fig. 6).



Figs 1–2. **1.** SDS-PAGE profile of *P. epiclitum* ES antigen. **2.** Western blot analysis of ES antigen.



Figs 3–4. **3.** Polypeptide profile of purified ES fractions. **4.** Polypeptide profile of F3 fraction (low, medium and high molecular weight) (low molecular weight).



Figs 5–6. **5.** Western blot analysis of F3 fraction (low molecular weight) with hyperimmune sera. **6.** Western blot analysis of F3 fraction (low molecular weight) with natural sera.

Table 1. Comparative two way analysis of microscopy and indirect-ELISA results

Faecal examination	Indirect-ELISA		Total
	Positive	Negative	
Positive	63 (True Positive)	21 (False Negative)	84
Negative	26 (False Positive)	148 (True Negative)	174
Total	89	169	258

Considering the rapid degradation of high molecular weight proteins even at ultra low temperature (-80ºC) storage and cross-reactivity of medium and high molecular weight immunodominant bands with *F. gigantica*, the low molecular weight antigenic fraction (F3) containing 11 kDa immunodominant band was selected for its use in indirect ELISA. Optimized ELISA was used to screen 258 bovine sera samples for antibodies against 11 kDa low mol wt. antigenic fraction of *P. epiclitum*. A total of 63 samples out of 84 positive were detected positive for *P. epiclitum* antibodies by ELISA. Also, 26 negative samples (out of 174) were detected as false positive. Based on the results, the sensitivity and specificity of ELISA was calculated as 75.0% and 85.0%, respectively (Table 1). The percent positive and negative predictive values were found to be 70.78% and 87.57%, respectively. The low sensitivity of the test may be due to low antibody response of the animals against chronic infection. The specificity of the test seems to be reasonably good, but it needs to be tested with sera of animals harbouring infection in the pre-patency phase. The potential of immunodiagnostic assays for early detection of helminth infections have been reported to be convulsed with low specificity and cross-reactions due to shared common antigenic epitopes, especially in trematodes (Ghosh *et al*. 2005). Previous studies conducted by other workers also recorded low sensitivity and specificity using somatic and/or ES antigens from adult flukes (Anonymous 2004, Kaur *et al*. 2009).

The findings of present study suggest that the low molecular weight fraction of *P. epiclitum* may be used for the immunodiagnosis of *Paramphistomum* infection in ruminants. However, concerted efforts and further studies are required to rule out the cross-reactivity of 11 kDa polypeptide of *P. epiclitum* with other common helminths infecting ruminants.

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