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Evaluation of Immunoblotting Technique for the Diagnosis of Somatic Antigens of *Paramphistomum cervi* Isolated from Local Cattle Breeds of Kashmir Valley

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Abstract: Paramphistomosis is a parasitic infection of the domestic and wild ruminants caused by various species of genus *Paramphistomum* belonging to the family Paramphistomidae. The infection with these amphistomes is frequently seen in the native cattle of Kashmir. When immature, the flukes live in the small intestine and abomasum, from where they migrate to the rumen and become adults. The antigenic profile of whole worm extract of *Paramphistomum cervi* was revealed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting using sera from cattle naturally infected with *P. cervi* as well as by sera from cattle infected with *Fasciola gigantica*, *Dicrocoelium dendriticum* and hydatid cyst to check cross reactivity. SDS-PAGE of whole worm extract revealed the presence of many protein bands with molecular weights ranging from 12 to 100 kDa. The major bands appeared at 66, 52, 31, 26, 22 and 12 kDa. Western blot analysis of these proteins showed the presence of six major antigenic bands which were recognized by serum of individual cattle naturally infected with *P. cervi*. These antigenic proteins had molecular weights ranging from 15 to 100 kDa. One antigenic protein with a molecular weight of 90 kDa was found to give a consistent reaction with sera from all infected cattle. This suggests 90-kDa protein can be specific diagnostic antigen for Paramphistomosis in Kashmir.

Key words: Paramphistomum · *Paramphistomum cervi* · Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) · Immunoblotting/Western blot

INTRODUCTION

Paramphistomes or stomach flukes are conically shaped digenetic trematode parasites belonging to the super family, Paramphistomidae [1]. The immature flukes of this parasite live in the small intestine and abomasum from where they migrate to reticulum and rumen and mature into the adults there. They are fleshy, pear shaped, measuring 5–12 mm (length) × 2–4 mm (width) and are pink or light red in colour, Juvenile fluke are however smaller (1–2 mm long). Paramphistomosis has been a neglected trematode infectious disease in ruminants, but has recently emerged as an important cause of productivity loss, that cause high morbidity and mortality and by affecting health, production and reproduction of ruminants particularly in young stock. Older animals can however develop resistance but may still harbor numerous adult flukes in the rumen and reticulum without showing overt symptoms, however in case of heavy infection, damage to the rumen has been recorded in the form of unthriftiness, emaciation, lower feed conversion rate, decrease milk yield and reduction of fertility [2- 4]. In case of acute infections, large number of immature flukes while migrating through the intestinal tract causes acute parasitic gastroenteritis especially in young ruminants [5, 6]. A transitory diagnosis of paramphistomosis is based on the history and clinical signs of the disease. Further confirmation can be obtained by examining the fecal samples for the presence of parasite eggs. However, this method is unreliable because the parasite eggs are not found during prepatent period and hence often results in misdiagnosis. More ever this method lacks sensitivity,

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especially in light infections or during subclinical disease [7, 8]. Early diagnosis of paramphistomosisis necessary for rapid treatment before irreparable damage to the rumen and bile duct occurs [9]. Different immunodiagnostic tests such as ELISA, Immunofluorescence assay etc. have been used in the early immune diagnosis of paramphistomosis, but they have some disadvantages, such as cross reactions with other trematodes, leading to false positive results [10]. In recent years, SDS-PAGE and Western blot procedures have created a new era in immunodiagnosis and greatly reduced cross reactions. Over the last two decades various studies to identify and characterize proteins of immunological significance have been carried out, especially the candidates for immunodiagnosis or vaccination in parasitoses [11- 16]. The identification and characterization of proteins in case of parasites is initiated by studying the antigenic profiles with the help of SDS-PAGE and immunoblotting. Thus in the present study, the whole worm extract of adult *P. cervi* has been analyzed for determination of antigenic profile using the SDS-PAGE and immunoblotting technique with the sera of the cattle naturally infected with *P. cervi*.

MATERIALS AND METHODS

Collection of Parasites: Adult *P. cervi* were collected from the rumen of naturally infected cattle slaughtered for consumption at the local slaughter houses. They were washed 3-4 times with phosphate buffer saline (PBS) and used immediately for antigen preparation or stored in the laboratory at -20°C until used.

Identification of the Parasite: The worms were fixed in formaline, stained with aceto-alum carmine, dehydrated in series of ascending grades of ethanol, cleared in xylene, mounted in DPX. The species were then identified morphologically according to Soulsby [17].

Antigen Preparation: The whole worm extract (WWE) was obtained by homogenizing the adult parasites in 0.01 M phosphate buffer saline (PBS), pH 7.2, containing 10 mM Tris–HCl, 150 mMNaCl, 0.5% Triton X-100, 10 mM EDTA and 1 mM phenyl methyl sulfonyl Floride (PMSF) using electrical homogenizator. Suspension was then centrifuged at 4°C at 10,000 g for 30 min and the supernatant was collected and preserved at -70°C until used. Protein concentration was determinedby Lowry *et al*. [18].

Collection of Serum Samples: Twenty paramphistomosis sera were obtained from cattle with confirmed *P. cervi* infection using a standard parasitological method like stool examination and ELISA. To examine the cross reactivity with other parasites, 10 serum samples were collected from cattle infected with fasciolosis, *Dicrocoelium dendriticum* and hydatid cyst. Negative control sera were collected from 10 normal cattle whose stool samples at the time of blood collection contained no parasite eggs. Positive sera were pooled by combining equal volumes of proven paramphistomosis sera. These pooled as well as individual sera were used in Immunoblotting analysis method.

SDS–PAGE Analysis: Somatic antigens of these flukes were separated by SDS-PAGE as described by Laemmli [19]. Gels were stained with 0.05% Coomassie brilliant blue and silver staining. The molecular weights of proteins were determined by comparing their migration distance against that of a known molecular marker.

Antigenic Analysis by Western Blotting: Antigenically active components among the resolved bands in SDS-PAGE were detected by Western blotting. After SDS-PAGE, the proteins were transferred electrophoretically onto a nitrocellulose sheet using a transfer blot apparatus [20]. Each blotted nitrocellulose membrane was cut into strips and incubated in a blocking solution (5% skimmed milk in Tris buffered saline (TBS) pH 7.4 containing 0.05% Tween 20) at room temperature for 2 h. Thereafter, the strips were incubated in serum samples, diluted to 1:100 with 1% skimmed milk in TBS, pH 7.4, containing 0.05% Tween 20, at room temperature for 2 h. After washing with TBS to remove unconjugated antibodies, each strip was then incubated in peroxidase- conjugated rabbit anti-bovine immunoglobulin G diluted to 1:4000 with 1% skimmed milk in TBS, pH 7.4, containing 0.05% Tween 20, at room temperature for 1 h. After washing with TBS, the color reaction was detected by using specific substrate 3,3'- Diaminobenzidine(DAB) atroom temperature for 3–5 min until the bands appeared.

RESULTS

Morphological identification of *P. cervi* was carried out on the basis of size and shape of fluke and position of anterior and posterior sucker. In the present study, most of the species were of *P. cervi,* which were found mainly

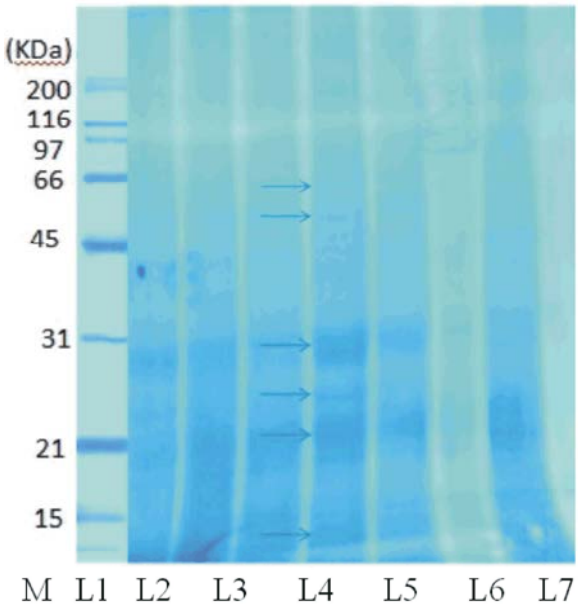

Fig. 1A: Commassie Brilliant Blue stained protein Profile of whole worm extract of *Paramphistomum cervi* from different bovine Rumens (lane L1-L7); Molecular marker (lane M).

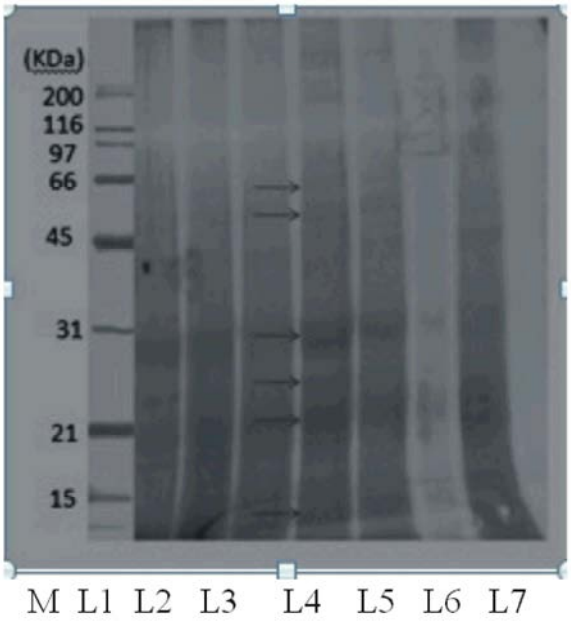

Fig. 1B: Silver stained protein profileof whole worm extract of *Paramphistomum cervi* from different bovine Rumens (lane L1-L7); Molecular marker (lane M).

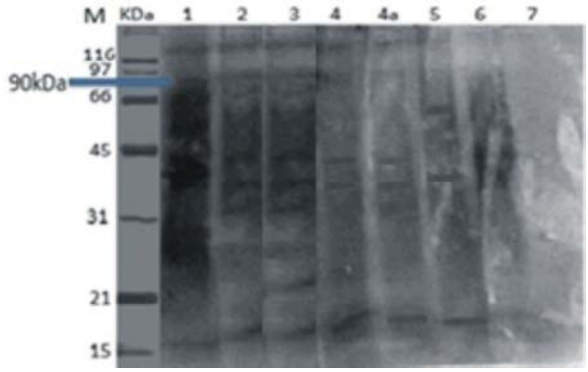

Fig. 2: Western blot analysis of somatic antigens of *Paramphistomum cervi* against serum samples M: Molecular weight marker, 1-3: Positive serum of amphistomes, 4 and 4a: Positive serum of *Fasciola gigentica,* 5: Positive serum of *Dicrocoelium dendriticum*,6: Positive serum ofhydatid cyst,7: Negative serum.

in the rumen and were light pink in color with a sucker at the tip of the cone and another sucker ventrally at the posterior end.

By means of SDS–PAGE with Commassie Brilliant Blue stain and silver staining method, *P. cervi* whole worm extract showed many protein bands with molecular weights ranging from 12 to 150 kDa at a concentration of 8 ìg/ml. The major bands appeared at 66, 52, 31, 26, 22 and 12 Kda (Fig. 1A and 1B). Sera of individual cattle with *P. cervi* infections reacted with six major antigenic proteins with molecular weights ranging from 15 to 100 kDa (Fig. 2).One distinct immunogenic band at 90 kDa was found to react with all of the sera from infected cattle with clinically diagnosed and parasitologically confirmed paramphistomosis. The specificity of the 90 kDa was studied by comparing their activities of *P. cervi* WWE with sera from *P. cervi* infected cattle, normal controls and from cattle infected with other closely related parasites (*Fasciola gigantica*, *Dicrocoelium dendriticum* and hydatid cyst). There was no positive band detected at 90kDa when using sera from control animals and animals infected with the other two parasites and hydrated cyst. Hence, we believe that 90 kDa antigen is quite specific for *P. cervi*.

DISCUSSIONS

Up to now, parasitological techniques were used for diagnosis of paramphistomosis which were based on the detection of eggs in fecal samples. However, parasitological diagnosis is only possible at about 12–16

weeks after infection when the parasite matures and begins to release eggs, while the symptoms occur as early as 2 weeks after infection. Further the parasitological examination often leads to misdiagnoses of the disease, because of the misjudgment of eggs in the fecal samples. Hence, a more reliable and convenient methods was necessary for diagnosing the infection. It was believed that serological method would be a better method to detect the early infection, which involves the analysis of antibody responses to fluke antigens. In order to do this, an antigenic profile of the parasite should be known. Our investigation is to demonstrate antigenic profile of adult *P. cervi* from a whole worm extract (WWE) by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting using sera from cattle naturally infected with *P. cervi, Fasciola gigantica, Dicrocoelium dendriticum* and hydatid cyst. The pattern and molecular weight range of the protein profile of *P. cervi* WWE obtained from SDS–PAGE in this study was similar to that of other closely related trematode parasites. For example [21] reported that *F. hepatica* exhibited prominent bands in WWE with the range of 14–94 kDa. Similarly [22] reported that the somatic extract of adult *F. gigantic* comprised many protein antigens ranging from 12 to 95 kDa. Another study of *F. gigantica* revealed that the somatic extract of adult *F. gigantica* consisted of more than 22 protein bands ranging from less than 14.4 to more than 94 kDa [23]. Similarly [24] showed 8 and 5 protein bands in somatic antigens with molecular weight ranging from 25.5-48 and 27-57.6 kDa in *F. hepatica* and *F. gigantica*, respectively. It has also been reported by [25] that 7 protein bands of 16 to 62 kDa molecular weight are found in somatic extract of *F. gigantica*. Moreover, 8 and 11 diagnosable somatic proteins band were shown for *F. hepatica* and *F. gigantica* with molecular weight ranging from 18-62 and 18-68 kDa, respectively [26].

Furthermore [27] showed that the sera from both sheep and cows experimentally infected with *F. hepatica* recognized the protein bands of 12–156 kDa. Likewise [28] revealed a strong reactivity of all infected sheep sera against somatic antigens at 20–23 kDa and excretory–secretory (ES) antigens of *F. hepatica* at 23–27 kDa. Hence, these antigens were suggested for use in the diagnosis of sheep fasciolosis. In addition [29] demonstrated that sera from both *F. hepatica* infected horses and pigs recognized protein of 14– 19 kDa, 22–30 kDa, 35–37 kDa and 40–42 kDa in crude somatic and ES antigens. They suggested that the 22–33 kDa proteins could be the most suitable candidate antigens for use in the immunodiagnosis of fasciolosis in horses and pigs. Another study of *F. hepatica* reported that the ES antigens of *F. hepatica* were composed of more than 11 protein bands of which five components were detected by human fasciolosis sera, with molecular weights of 12.4, 16.4, 19.4, 25 and 27 kDa. Only the 25 and 27 kDa components were suggested to be sensitive and specific enough for the diagnosis of human fasciolosis [30].

In the present study, the electrophoretic analysis of *Paramphistomum cervi* somatic antigen gave 14 distinct protein bands (ranging from 12KDa to 100KDa). The immunoblotting analysis of *P. cervi* has revealed six major positive antigenic bands, of which the 90 KDa antigen reacted with sera from all infected cattle. As well, this antigen did not react with sera from cattle infected with other parasites, i.e, *Fasciola gigantica, Dicrocoelium dendriticum* and hydatid cyst. These results are relatively different with that obtained by Salib *et al*. [31] who found 14 distinct protein bands by electrophoretic analysis of *Paramphistomum* somatic antigen (ranging from 11.5 KDa to 174 KDa of protein molecular weight 11.5, 13.5, 19, 25, 29, 46, 52, 63, 66, 72, 87, 105,120 and 174 KDa and further the Western blotting analysis of *Paramphistomum* somatic antigens with Serum samples from hyper immune rabbit and individual cattle and buffaloes with *Amphistomes* infection by Salib *et al*. [31] revealed immune-dominant polypeptides of molecular weights 27, 39, 58, 63,71 and 87 KDa and one distinct immunogenic band at 63 KDa was found due to the reaction of *Paramphistomum* somatic antigen with all sera from infected cattle and buffaloes which was not consistent with our results. However, the results were in consistent with Meshgi *et al*. [32] who revealed the presence of 10 protein bands of molecular weights ranging from 25-120 by electrophoretic analysis of somatic antigens of *Amphistomes* and demonstrated five major proteins of molecular weight ranging from 50 to 100 KDa by Immunoblotting with serum of cattle naturally infected with mixed amphistomes. They also found that 90-kDa protein can be specific diagnostic antigen for mixed amphistomes in Iran. Also Arora *et al*. [33] recorded that *Paramphistomum epiclitum* somatic antigen contained protein bands ranging from 14.9-95.5 KDa and reported two lower molecular weight protein bands of molecular weight 37.6 and 39.8 as a most common specific antigen for *P. epiclitum,* while Anuracpreeda *et al*. [34] by SDS-PAGE of whole worm extract of *P. cervi* showed 26 distinct bands of proteins with molecular weight ranging from 11.5 to 200 kDa. and found that five major antigenic

bands of molecular weights ranging from 23 to 116 Kda were recognized by serum of cattle infected with *P. cervi* and reported that 52 KDa antigenic protein band is most specific band for the diagnosis of *P. cervi.* These results show diversity between somatic protein bands of various species of *Paramphistomum.* The differences in the results of [31, 32, 33. 34] and the current results can be attributed to single and mixed amphistome infections in the cattle. Besides the difference can be attributed to the subsequent ecological and geographical parameters.

CONCLUSION

The wetern blot analysis of *P. cervi* showed many protein bands ranging in molecular weight from 15 to 100 kDa. It was seen that many of these bands were common among different parasites. However, a unique band of molecular weight 90 kDa was found to give a consistent reaction with the sera of all the cattle infected with *Paramphistomum cervi*. This shows that 90 kDa protein band can be used in diagnostic purposes and can serve as a potential vaccine candidate. However, this needs further trials.

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