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Original Article

Determination of Diagnostic Antigens in Cattle Amphistomiasis Using Western Blotting

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Abstract

Background: Mixed infection with amphistomes seems common in native cattle of Iran. The aim of this study was to determine diagnostic antigens in cattle mixed amphistomiasis.

Methods: Specific antigens of *Cotylophoron cotylophorum*, *Gastrothylax crumenifer* and *Paramphistomum cervi* (mixed infection), the most common species, were collected from cattle was determined. Adult trematodes were collected from the rumen of naturally infected cattle at meat inspection. After their homogenization and centrifugation, somatic antigens were prepared and analyzed by SDS-PAGE. Specific antigens were determined by western blot with homologous and heterologous sera. SDS-PAGE of whole worms extract was performed at different concentrations and subsequent gels staining. Immunoblotting analysis using sera from cattle naturally infected with amphistomes, *Dicrocoelium dendriticum*, *Fasciola* spp. and hydatid cyst was performed.

Results: Electrophoresis analysis of somatic antigens revealed the presence of 10 and 21 protein bands at 4 µg/ml and 8 µg/ml with molecular weights ranging from 25-120 and 25-150 kDa, respectively. The best result was taken at 8 mg/ml concentration. Although western blot of these proteins demonstrate 5 major antigenic polypeptides ranging from 50 to 100 kDa which were recognized by serum of cattle naturally infected with mixed amphistomes.

Conclusion: Ninety-kDa protein can be specific diagnostic antigen for mixed amphistomiasis in Iran.

Keywords: Amphistomiasis, Cattle, Antigens, Western blotting

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Introduction

Paramphistomiasis has been a neglected trematode infectious disease in ruminants, but has recently emerged as an importance cause of productivity loss (1). Adult fluke that lives in rumen and reticulum of ruminants does not cause serious problem, but massive number of immature paramphistomes can migrate through intestinal tract causing acute gastroenteritis in the small intestine with high morbidity and mortality rate especially in young animals (2-4). Mixed infection is common in ruminant of Iran and 3 species were identified in cattle, sheep and goats (5, 6). In a more comprehensive morphometric study 10 species including: *Paramphistomum cervi*, *P. gotoi*, *P. gracile*, *P. microbothrium*, *Cotylophoron cotylophorum*, *Gastrothylax crumenifer*, *G. compressus*, *Carmyerius spatiosus*, *Calicophoron papillosum*, *Orthocoelium scoliocoeium* were identified in ruminants of Iran (7). Meanwhile it was shown that *Bulinus truncatus* is intermediate host of *P. microbothrium* in Khuzestan, south Iran (8). Despite of this, no information is available in the literature on the other aspects of infection including diagnosis, clinical signs and treatment of the infection in Iran. Coproscopic analyses, often results misdiagnosis is not wholly reliable, and could not be used in early diagnose which is essential for prompt treatment before irreparable damage to the rumen and bile duct (9).

In the present study, the attempts were made for determination of diagnostic antigens in mixed cattle amphistomiasis.

Material and Methods

Antigen Preparation

Adult amphistomes including: *Cotylophoron cotylophorum*, *Gastrothylax crumenifer* and *Paramphistomum cervi* were collected from the rumen of naturally infected cattle at meat inspection and were

transferred immediately to the laboratory and was kept in -20°C until used. They were washed 3-4 times with phosphate buffer saline (PBS). Adult flukes were homogenized in 2 ml of PBS (0.01 M, pH=7.2) containing phenyl methyl sulfonyl fluoride (PMSF 0.05 mM) using a tissue grinder for obtaining whole worm extract. 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 determined by Bradford method (10).

Serum samples

Twelve amphistomes sera were obtained from native cattle infected only with mixed amphistomes at necropsy (gold standard). To examine the cross reactivity with three other common parasitic infections of cattle in Iran including *Dicrocoelium dendriticum*, *Fasciola* spp. and hydatid cyst, 7 serum sample representative of each single infection and ten from non infected cattle according to necropsy findings as negative control were collected. Positive sera were pooled by combining equal volumes of sera of cattle harbored amphistomiasis. This pool as well as individual sera was used in western blotting analysis.

SDS-PAGE and Immunoblotting

SDS-PAGE of somatic proteins was carried out using a MINI-Protean III cell (Bio-Rad) at 70 constant voltages for 110 min according to the method of Laemmli (11). Electrophoresis of samples was performed in different concentration of antigens. For size estimation, a protein ladder marker, 10-200 kDa (Fermentas, SM0661) and a pre-stained protein standard broad range, 10-160 kDa molecular weight (Fermentas, SM0671) were used for SDS-PAGE and immunoblotting respectively. Proteins were stained with Coomassie blue. Gel antigens were electrophoretically transferred to nitrocellulose sheets for im-

munoblotting according to the procedure described by Towbin et al. (12). Electro-transferred of proteins onto nitrocellulose membrane were performed by Bio-Rad Trans-Blot Cell system at 40 constant voltages during over night. Afterwards, the transferred proteins were blocked with 3% BSA powder in Tris-buffered saline with 0.05% Tween 20 (blocking solution) at room temperature for 60 min. Nitrocellulose strips were washed (3×10min). The strips were incubated for 60 min with serum samples, diluted 1:1000 and washed again for 3×10 min. The strips were next incubated with horseradish peroxidase conjugated rabbit anti-bovine (1:10,000) for 1 h at room temperature. After incubation, the strips were washed as before in PBS 0.1% Tween 20. 3, 3'-diamino benzidine tetra hydrochloride substrate was in 25ml of PBS with 10 µl of 30% H₂O₂ added for exactly 2-5 min at room temperature. Bands were visible within 5-10 min. The color development was stopped by rinsing de-ionized water.

Results

Our findings showed that the best soluble extract concentration of antigens for SDS-polyacrylamide gel electrophoresis is 8 µgr/ml SDS-PAGE analysis of the whole amphistomes extracts at concentrations of 8 µgr/ml and 4 µgr/ml showed protein bands of various intensities ranging from 21 and 10 bands with a molecular mass between 25-150 and 25-120 kDa respectively (Fig. 1).

In the western blotting of whole worm extracts of amphistomes 5 major peptide bands ranging from 50 to 100 kDa were recognized by serum of individual cattle naturally infected with mixed amphistomes (Fig. 2, Lan A1-A3). No protein band was observed in non infected control animals sera (Fig. 2, Lan N), although 4, 3 and 2 bands occurred using infected *D. dendriticum*, *Fasciola* spp. and hydatid cyst sera respectively (Fig. 2, Lan D, F, H) but at 90 kDa molecular weight no bands when using sera from animals infected with the other three parasites.

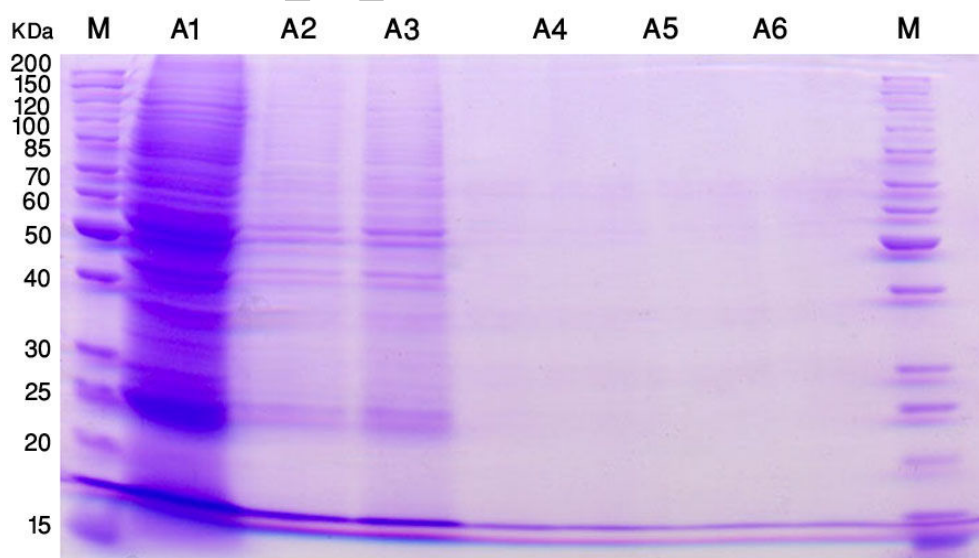


Fig.1. SDS-PAGE analysis of somatic antigens of amphistomes of different concentration. M: Molecular weight marker (Fermentas=SM0661) A1-A6: Different concentration of somatic antigen (8, 4, 4, 2, 2, 1 µgr/ml, respectively)

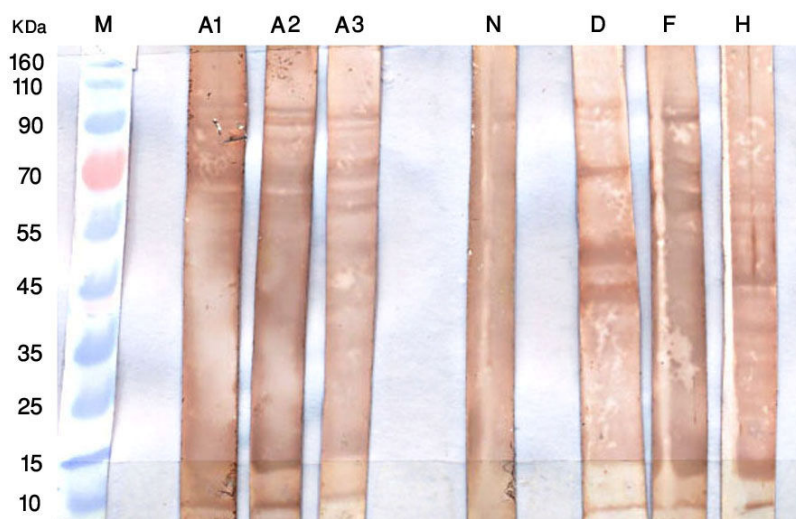


Fig. 2. Western blot analysis of somatic antigens of amphistomes against serum samples M: Molecular weight marker (Fermentas=SM0671), A1-A3: Positive serum of amphistomes, N: Negative serum, D: Positive serum of *Dicrocoelium dendriticum*, F: Positive serum of *Fasciola* spp., H: Positive serum of hydatid cyst.

Discussion

Paramphistomiasis still is a neglected trematode infectious disease of ruminants in Iran, more or less, similar to other parts of the world (1). Beside the identification of 3 species of amphistomatide in sheep and cattle (5, 6) and 10 species in ruminants in Iran (7) no other information exist on the prevalence, clinical signs, diagnosis and treatment of infection, although Arfaa (8) showed *Bulinus truncatus* as intermediate host of *Paramphistomum microbothrium*. In a pilot study on the prevalence of paramphistomiasis in 3 different climatic conditions of Iran e.g. north of Iran along the Caspian Sea with moderate and humid weather and considerable rainfall (zone I). Mountain plateau zone with semi-dry weather (zone II) and south west along the Persian Gulf with subtropical and humid weather: 45%, 4% and 10% of local cattle harbored amphistomes respectively (unpublished data). In all studies carried out in Iran mixed amphistomes infection has been common, a pattern supporting the findings of other workers in Asia including

India and China (4, 9). Although at present diagnosis of amphistomiasis in live animals still depends on fecal detection egg, but this method is not reliable enough, because of misjudgment of the egg in the feces and the absence of eggs during migrating phase of trematode to small intestine, when eggs have not been produced. This call for presentation immunodiagnostic method is complementary to coproscopic analysis.

Anuracpreeda et al. (1) 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. In the present study 10 and 21 bands were obtained at protein concentration of 4 µgr/ml and 8mg/ml, respectively, with molecular weight of 25-120 kDa due to paucity of information on amphistomes protein profile a comparison will be made between the latter and closely related trematode e.g. *Fasciola*. Allam et al. (13) 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. Similarly, Upadhyay and Kumar (14) reported 7 protein bands of 16 to 62 kDa molecular weight for somatic extract of *F. gigantica*. Other workers showed protein bands ranging from 14 to 94 and 12 to 95 kDa, for whole worm extracts of *F. hepatica* and *F. gigantica*, respectively (15, 16). 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 (17). These results show diversity between somatic protein bands of *Fasciola*, similar to the two existing reports on amphitomes. These differences can be attributed to single and mixed amphistome infection in Anuracpreeda et al. (1) and present study. Subsequently ecological and geographical parameters could be taken into consideration.

In immunoblotting of sera from mixed cattle amphistomiasis, were found 5 major immunogenic proteins bands for whole worm extracts with molecular weight ranging 50 to 100 kDa. In Western blotting of somatic antigen of *F. gigantica* against infected human sera, bands less than 14.4 to more than 94 kDa molecular weight were specific for humans (18). Subsequently a 38 kDa component was suggested as useful diagnostic antigen for human fasciolosis. Using the same approaches, on immunoblotting of *P. cervi*, 5 major protein antigenic bands were shown of which the 52 kDa antigen reacted with sera from all infected cattle, but not with sera from cattle infected with two closely related parasites, i.e. *F. gigantica* and *strongyloides* (1).

Conclusively, in our findings similar to Anuracpreeda et al. (1), 5 immunodominant antigenic bands reacted with sera in all cattle harbored mixed amphistomiasis using western blotting with molecular weight of 50 to 100 kDa. Although 4 bands were reacted with three other closely prevalent ruminants parasites in Iran i.e. *F. hepatica*,

D. dendriticum and hydatid cyst, but a 90 kDa band seems to be specific for diagnosis of mixed cattle amphistomiasis in Iran.

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