Determination of Immunodominant Antigens of Dicrocoelium Dendriticum by Hyperimmune Sera

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Abstract Dicrocoeliasis caused by the small liver fluke Dicrocoelium dendriticum (syn. D.lanceolatum), which live in the bile ducts and gall bladder of wild and domesticated mammals, particularly livestock farming. Parasitological methods are not reliable enough for diagnosis of this trematode infection. In the present study, the sera from rabbits immunized with somatic and excretory-secretory antigens of D.dendriticum were assayed with immunoblot technique. Adult worms were collected from infected livers of sheep at a local abattoir. The excretory-secretory product and somatic antigens were prepared through homogenization and incubation of trematodes, respectively. Polyclonal antibodies were raised in rabbits after inoculation of the two antigens with emulsified Freunds adjuvant followed by booster injection. Comparative study between D.dendriticum antigens and those of Fasciola, cysticercus tenuicollis and hydatid cyst fluids antigens was carried out. SDS-PAGE of antigens was performed and for immunoblotting analysis, hyperimmune sera were obtained from immunized rabbits of case and control groups. Immunoblot analysis of rabbits antisera against somatic and excretory-secretory antigens of D.dendriticum were showed six and four protein bands ranging from 25 to more than 170 and 25 to 70 kDa molecular weights, respectively. However cross-reaction with other specific proteins was observed in Fasciola excretory-secretory, hydatid cyst and cysticercus tenuicollis fluid antigens. The results demonstrated that the 25-27 kDa proteins were strongly recognized by both hyperimmune sera and was common in both somatic and excretory-secretory antigens. According to the results of this study performed with two antigens of D.dendriticum shows that the 25-27 kDa polypeptide as immunodominant protein, could be considered for the immunodiagnosis of dicrocoeliasis and it probably induces protective immunity against Dicrocoelium infection.

Keywords Immunodominant Antigen, Dicrocoelium Dendriticum, Westernblot

1. Introduction

Dicrocoeliasis mostly remains clinically undiagnosed, because of its generally subclinical future and diagnose of infected animals are fundamentally due to isolating adult flucks from the liver at necropsy or detection of eggs at coprological examination (Otranto and Traversa, 2002). It is widely distributed in Iran and is common in wild and domestic ruminants of Iran where 16% of cattle, 39.4% of sheep, 70.1% of goats and 11.1% of buffalos have been found to be infected (Esami, 2008). There has also been an increase in the number of case reports especially ruminants in Iran. D.dendriticum with worldwide distribution causes significant economic losses due to condemned infected livers as well as digestive disorders that caused growth delay, reduced milk production and decreased weight (Manga-Gonzalez et al., 2004; Wolff et al., 1984). It has a complex and triheteroxenous life cycle because it involves more than 99 land mollusks and at least 21 ant species as the first and second intermediate hosts (Otranto and Traversa, 2002). The diagnosis is based on recovering adult flukes at necropsy or postmortem inspection. Coprological examination is the most commonly used method for isolation of egg in live animals as well (Campo et al., 2000; Rehbein et al., 2002). The latter method which is commonly used is not reliable enough to diagnose the prevalence especially the intensity of infection.

Therefore, a variety of diagnostic methods have been suggested to the study of D.dendriticum infection such as, ELISA (Revilla-Nuin et al., 2005; Wedrychowicz et al., 1995), agar gel precipitation, counterimmunoelectrophoresis and passive haemagglutination tests (Jithendran et al., 1996) have been used to diagnose the infection and to reveal the present antibodies against D.dendriticum in infected animals.

Meanwhile different antigens such as somatic extract of adult trematodes (Jithendran et al, 1996) and crude extract of eggs (Bode and Geyer, 1981) were used for immunological diagnosis of dicrocoeliasis. Besides, the bile antibody responses of naturally infected cattle against somatic, excretory-secretory (E/S) and surface antigens of adult D.dendriticum were assessed (Wedrychowicz et al., 1995; Wedrychowicz et al., 1996).

The present study reveals soluble somatic and
excretory-secretory proteins and the identified immunodominant antigen of *D. dendriticum* as alternate sources of antigens which bind to the rabbit hyperimmune sera.

2. Material and Methods

2.1. Antigen Preparation

Adult trematodes of *Dicrocoelium dendriticum* were collected from the livers of infected sheep at necropsy from a local abattoir. The worms were washed extensively in phosphate buffered saline (PBS, pH: 7.3) and soluble extracts were prepared according to Farrell et al., (1981). Briefly, adult flukes homogenized in a Ten Brock tissue grinder at 4˚C, homogenate were centrifuged at 12,000 × g for 30 min and supernatant fluids in the presence of protease inhibitors, phenylmethylsulfonyl fluoride (PMSF, 0.05mM) were collected as Dd So. Excretory/secretory antigens were prepared according to Gonzalez-Lanza et al., (2000) with slight modifications. Adults trematode incubated for 8 h in PBS (containing PMSF) at 37˚C, the preparation was centrifuged at 10,000 × g for 30 min at 4˚C. The supernatant thus collected was designed as Dd E/S Ag. The protein concentration of antigens was measured as described by Bradford method (Bradford, 1976). All antigens were preserved at -70˚C until used.

2.2. Hyperimmune Sera Preparation

Six native 6-7 months rabbits were divided randomly into three groups, two rabbits in each group. Four rabbits were immunized sub-cutaneously with *D. dendriticum* somatic and excretory-secretory antigens. The antigens (0.5 ml) were mixed with an equal volume of Freund’s complete adjuvant and injected 4 rabbits (two rabbits for each antigen). Subsequently, two booster immunization with Freund’s incomplete adjuvant were administered at intervals of 2 weeks. Two rabbits as control were injected with saline adjuvants. Blood samples from each rabbit in experiment and control group were obtained intracardially 1-week after last inoculation. All sera samples stored at -20˚C.

2.3. SDS-PAGE and Westernblot Analysis

SDS-PAGE of antigens was carried out according to the Laemmli with an 12% resolving gel and a 5% stacking gel (Laemmli, 1970). The antigens were electrophoretically transferred from the gel onto a nitrocellulose sheets by the procedure of Towbin et al., (1979) with slight modifications. A prestained protein marker at range 15-170 kDa Molecular weight (Fermentase-Chemical, SM0671) were used for calibrating the gel. The electrophoretic transfer was performed at 30 V for 12 h in a Mini Trans Blot Cell (Bio Rad). Non-specific sites were blocked with 3% skim milk for 1 h. The nitrocellulose sheet was washed three times in PBS-Tween20 and was incubated for 1 h at room temperature with a 1:200 dilution of hyperimmune sera. After repeated washing, the sheets were incubated in peroxidase labeled antibodies conjugated at a dilution of 1:1000 for 1 h at room temperature. Finally the specific polypeptides were visualized by substrate buffer containing DAB (3, 3’- diamino benzidine tetra hydrochloride, Sigma) chromogen. The bands were visible within 10 min.

3. Results

In this study, immunodominant antigens of *D. dendriticum* were determined by westernblot, in order to antibody detection using rabbit's sera against whole somatic and excretory-secretory antigens. The results of immunoblot both antigens of *D. dendriticum* and other antigens to rabbit's positive sera which injected with somatic and excretory-secretory antigens *D. dendriticum* are shown in Figures 1 and 2, respectively.

Western blot analysis revealed present of 6 components peptide 25-27, 40, 55, 73 and more than 170 kDa (consisting 2 peptides) bands in somatic rabbits antisera (Figure 1, Lane 1) and detected the same 6 protein bands against E/S rabbits antisera (Figure 2, Lane 1). Protein bands of 25-27, 45, 47, 55 and 25-27, 30, 55, 70 kDa were recognized by somatic (Figure 1, Lane 2) and E/S antigens (Figure 2, lane 2), respectively. This results shows that 25-27 kDa band were specific for rabbit antisera. In control group no protein band was formed, although 1, 2 and 1 protein bands were observed using *Fasciola* excretory-secretory, hydatid cyst and cysticercus tenuicollis fluid antigens (Figure 2, Lanes 4, 5, 6) which their molecular weight ranged from 60 to 70 kDa molecular weight.
The findings of the present study indicate that \textit{D.dendriticum} somatic and excretory-secretory antigens against sera from rabbits immunized with the same antigens, commonly polypeptide with 25-27 kDa molecular weights, in both antigens is an immunodominant protein for \textit{Dicrocoelium} infection.

4. Discussion

The faecal egg count, probably the most often used diagnostic method, is not considered to be an optimal indicator of liver fluke burdens, however direct correlations were found between the number of eggs and parasite burdens in both experimentally and naturally infected sheep (Campo et al., 2000; Rehbein et al., 2002). Generally, faecal examination is not a suitable method to determine the infection (Sanchez-Andrade et al., 2003). In comparison, coprological sedimentation and indirect ELISA indicated seropositivity of 86.2\% in examined sheep, whereas faecal prevalence was 6.7\%. Somatic (8), surface and E/S (Wedrychowicz et al., 1996) antigens of adult \textit{D.dendriticum} recognized by bile antibodies of naturally infected cattle. Wedrychowicz et al. showed that in ELISA method, surface
glycoproteins, surface proteins and excretory secretory antigens reacted with samples sera, 92%, 70% and 38-67%, respectively (Wedrychowicz et al., 1996).

Attempts have previously been made to develop an accurate specific antigen for \textit{D. dendriticum} infection (Revilla-Nuín et al., 2005; Simsek et al., 2006), because of epidemiology studies can be a suitable initial approach to improve control and prevention of helminthiasis such as dicroceliosis. On the other hand, the accurate diagnosis of helminthes infection has important implications for disease epidemiology, prevention and treatment.

In this research, antisera from rabbits immunized with somatic and E/S antigens of \textit{D. dendriticum} were used for specific protein detection by immunoblot analysis. Our findings showed a major polypeptide band of 25-28 kDa molecular weights in both antigens.

Simsek et al. using western blot of sera from naturally infected sheep, recognized 2 polypeptides with 98 and 205 kDa molecular weight in excretory-secretory antigen of \textit{D. dendriticum}. However, they reported 205 kDa band as specific protein (Simsek et al., 2006). In a similar study, somatic and excretory-secretory antigens of \textit{D. dendriticum} were studied by western blot method (Wedrychowicz et al., 1996). Immunoblot analysis using sera sheep infected with \textit{D. dendriticum} detected 8 major polypeptides from 24 to 205 kDa and 7 protein bands from 26 to 205 kDa molecular weight for somatic and excretory-secretory antigens, respectively. In addition, a specific polypeptide with apparent molecular weight of 130 kDa was detected, that could be used for \textit{D. dendriticum} diagnosis. Although results of Simsek et al., (2006) opposed Revilla-Nuín et al., (2005) our results coincide with Revilla-Nuín et al., (2005) who found 24 and 26 kDa bands in somatic and excretory-secretory antigens, respectively (Revilla-Nuín et al., 2005; Simsek et al., 2006). This low molecular weight polypeptide in both excretory-secretory product and somatic antigen as an antigenic protein probably increases the immunogenicity and can have potential diagnostic and protective value.

In other trematodes including \textit{Schistosoma mansoni}, \textit{Paragonimus heterotremus} and \textit{Gastrothylax crumenifer}, the low molecular weight polypeptides were recognized dominantly as specific antigens. Ruppel et al. in immunoblotting of \textit{S. mansoni} antigen with sera of patients infected with schistosomiasis (Ruppel et al., 1985), found a 31 kDa protein band, whereas Indrawati et al. by this method for diagnosis of paragonimiasis, reported a 35 kDa polypeptide band which showed 100% sensitivity and specificity (Indrawati et al., 1991) and besides, Safiullah et al. in \textit{G. crumenifer} trematode, demonstrated a polypeptide with the range of <15-50 kDa molecular weight used for immunodiagnosis (Safiullah et al., 2000).

According to the results this study suggest that the 25-27 kDa protein band could be a diagnostic antigen for dicroceliosis, although, further researches are needed in order to determine the potential immunodiagnostic antigen by different serological procedures which can probably be used for vaccination against dicroceliosis.

\textbf{REFERENCES}


