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Identification of Some Immunogenic Proteins of *Fasciola gigantica* using Immunoblotting Technique

Ibtisam A. Goreish[1]; Williams[2], D. J.; McGarry[2], J.; Abdel Salam[3], E. B.; Majid[4], A. M. and Mukhtar[5], M.M.

(1) Central Veterinary Research Laboratories Centre, P. O. Box 8067(AlAmarat), Khartoum, Sudan.(2) Veterinary Parasitology, School of Tropical Medicine, Liverpool University, UK. (3) Department of Pathology, Faculty of Veterinary Medicine, University of Khartoum, P.O. Box 32, Khartoum North, Sudan. (4) National Centre for Research, Khartoum, Sudan. (5) Institute of Endemic Diseases, Faculty of Medicine, University of Khartoum, Khartoum, Sudan

ملخص البحث

أستخلصت مستضدات من جسد طفيل المتورقة العملاقة (*Fasciola gigantica*) ومنتجاتها الإخراجيه والإفرازية. حيث تم تحليلها لمعرفة البروتينات الممنعه و التى يمكن إستخدامها فى التشخيص المبكر و التحصين ضد مرض ابوكبيده. أسفرت تقنية الرحلان الكهربائي (SDS –PAGE) عن وجود عدة حزم بروتينية ذات أوزان جزيئية مختلفة في كل من جسد الطفيل ومنتجاتها الإخراجيه والإفرازية المختلفة.

عند الكشف عن الحزم البروتينية بواسطة الامصال الممنعة طبيعياً و معمليا باستخدام تقانة التخطيط الغربي (Western blotting) تم التعرف علي عدد من الحزم البروتينية اوزانها الجزيئية تراوحت بين27-30 كيلو دالتون. كما أيضا تم بواسطة امصال حيوانات مصابة بالمتورقة العملاقة التعرف علي حزم بروتينية كانت اوزانها الجزيئية 44, 50, 60, 100و120 كيلو دالتون من المنتجات الإخراجيه و الأفرازية لفترة 5-8 أسابيع. خلص البحث الي أن الحزم البروتينيه ذات الأوزان الجزيئيه 27-30 كيلو دالتون تحتوي علي الإنزيمات التي يمكن ان تلعب دور رئيس للنشاط الحيوى والمحفز للجهاز المناعي و التي يمكن تجربتها كلقاحات للسيطره علي المرض.

Summary

***Fasciola gigantica* antigens were extracted from the somatic (SO) and excretory – secretary (E/S) products and analyzed for detection of immunoreactive proteins that could be used for early diagnosis and development of a protective vaccine. SDS-PAGE resulted in protein bands of different molecular weights in both parasite products.**

The SO extracts and E/S products of *F. gigantica* were then probed by Western blotting technique using immune sera from naturally and experimentally infected cattle for identification of immunoreactive antigenic components. Polypeptides between 27 and 30 KDa were identified by the sera of all infected animals in both parasite products. Proteins of 44, 50, 60, 100 or 120 KDa were also detected in E/S products with 5 –8 weeks post infection antisera.

Introduction

Fasciolosis, a disease caused by the liver fluke *Fasciola*, is of cosmopolitan distribution and widely reported in cattle and sheep-raising areas. Two species of *Fasciola*; *Fasciola hepatica* and *F. gigantica*, are known to cause the fasciolosis in domestic animals and man (Solusby,1982).

In the Sudan and other tropical countries, fasciolosis is caused by *F. gigantica* with a seasonal transmission in ruminants. The disease is enzootic in certain parts of the sudan, particularly the White Nile State. The highest risk of transmission rate occurs between December and May with peak ova excretion between August and September; a period coinciding with the rainy season. These wet conditions are conducive for the development of *Fasciola* eggs and hence infections of the intermediate snail host *Lymnea natalensis (*Haroun et al, 1986).

It has been reported that *Fasciola* spp undergoes antigenic changes during their migration through the hepatic parenchyma. These changes are mainly related to their tegumental surface proteins (Bennett and Threadegold, 1975; Hanna, 1980; Hughes,

1987). Migration is facilitated by enzymes secreted in the intestine of the migratory fluke (ref.). These enzymes include several proteinases, e.g. Cysteine proteinase and cathepsin proteinase which are also produced by the adult fluke (Carmona *et al,* 1993; Collins *et al*, 2004). During migration, the parasite feeds on the hepatic tissue and blood (Dawes, 1961) and degrades the extracellular matrix and basement membrane of hepatic tissue (Berasain *et al*, 1997).
The cysteine proteinase may be involved in degrading host haemoglobin (Dalton *et al*, 1995, 2003; Brindley *et al*, 1997). Cathepsin proteinases is present as 28 KDa protein in SO and E/S products of the juvenile liver fluke. Nevertheless, the different proteinases of the parasite are exposed to the host's immune system with consequent onset of protective immune response during the migratory phase.

Cathepsin proteinase has long been observed with the invading juvenile fluke in different mammalian hosts (Movsesijan and Javanovic, 1975; Duffus and Franks, 1981). In addition, the tegumental surface components of the migratory fluke were also found to provoke an immune protective response (Davies and Goose, 1981). This was indicated by the high levels of protection induced by vaccination with cathepsin L proteinase against ovine fasciolosis (Piacenza *et al*, 1999).

The aim of this study was to determine which protein in E/S products and SO of *F. gigantica* is recognized by antibodies from naturally and experimentally *F. gigantica* infected cattle using Western immunoblotting technique. The identification of these antigenic proteins is highly required for early diagnosis of fasciolosis as well as a possible provision of a further knowledge on the mechanisms of resistance to the disease.

Materials and Methods

Collection of parasites:

Adult *Fasciola gigantica* worms were collected from the bile ducts of infected livers, washed 6 times in 0.01 M phosphate buffered saline solution (PBS; pH 7.2) and used to prepare the parasite products.

Serum samples:

Blood serum samples were collected from animals naturally infected with *F. gigantica* in Kosti-area, the White Nile State, Sudan; an area where fasciolosis enzootic. Confirmation of infection was based on detection of *F. gigantica* eggs in animal's faeces. Blood samples were collected from the jugular vein and serum was separated and stored at -20°C for further studies.

Cattle experimentally infected with 500 metacercariae of *F.gigantica*, were kept in the premises of the Central Veterinary Research Laboratories Centre, Sudan. They were bled from the jugular vein at a weekly interval and the serum was immediately separated and stored at -20°C until used.

Preparation of parasite proteins products:

Excretory/secretory products (E/S):

Collected *Fasciola gigantica* were washed six times in RPMI-1640 medium and then incubated (for….. at…..)in culture flasks containing RPMI-1640 medium with 2% glucose 30 mM Hepes and 25mg gentamycin per ml of medium (one worm per 2.5ml) at 37°C according to Dalton *et al*. (1995). Following the incubation of *Fasciola gigantica*, the culture medium was removed, centrifuged at 14900 g for 30 min. and the supernatant was analyzed for protein concentration according to Warburg and Christian (1941). The antigen was then aliquoted and stored at -20°C until used.

Somatic antigens (SO):

After collection of E/S antigens (the supernatant), the worms were separated and ground on dry ice using a pestle and mortar. Four volumes of PBS were added and the ground material was left to stand for 30 min. on ice, with occasional shaking. The soluble somatic extract was collected by centrifuging the homogenate at 5000g at 4°C for 15 min. After dialysis, the homogenate was centrifuged again for 5000X at 4°C for 15 min. Protein concentration was determinedaccording to Warburg and Christian (1941) and the antigen was aliquoted and stored at -20°C until used.

SDS- PAGE and protein transfer:

Fractionation of E/S and SO products was accomplished onto10% poly-lyacrylamide gel slabs containing SDS (Laemmli, 1970). The two protein preparations were diluted with equal volume of sample buffer (2% SDS and 0.1M dithiotreitol, Sigma chemical Co.) in trisbuffer (pH 6.8) and boiled 3 min. Fifteen micrograms of proteins of each of E/S and SO products were loaded in each lane of a separate gel. Samples from *F. hepatica* and Purified cycteine L-protease were also loaded in E/S gel. Molecular weight standard markers (BioRad, Hemel Hempstead, and Hertforshire) were loaded in one of the outside lanes of the gel. Samples were electrophoresed at 25 mA per gel (i.e. total 50 mA until the lowest molecular weight standard band had migrated to approximately 1cm from the bottom of the gel; about 60 min).

Western blots:

The electrophoresis-separated proteins were transferred onto immobilon- P-paper (Immobilon-polyvinylidene difluoride) in transfer buffer (20 mM Tris and 100 Mm Glycine buffer) containing 20% Analar methanol using a semi-dry blot transfer system for 30 min. (BioRad, UK).

Immunoblotting:

Immunoblotting of proteins was done using antigen sera of *F. gigantica* naturally and experimentally infected cattle. The immobilon-P paper was blocked for 30 min at room temperature with 4% skimmed milk in PBS. The paper was then washed twice with 0.1% Tween-20 /TBS (washing solution for 3-10 min each) and finally once for 30 min. The immobilon-P-paper was then cut into strips and each strip was incubated at room temp. With test or control serum samples diluted 1:100 in solution containing 5% skimmed milk powder in TBS/Tween-20 (incubation buffer) in plastic strip containers and incubated in a rocking platform for 1hr. After three washes with washing buffer, the strips were incubated with anti-bovine IgG conjugated with horse reddish peroxidase (HRP) in 0.1%/PBS, 4% skimmed milk at a dilution of 1:1000. Thereafter, the antigenic bands were visualized using DAB method (3, 3'-diaminobenzidine tetra hydrochloride). The reaction was stopped by rinsing with water for the brown bands to intense.

Results

The results of SDS-PAGE for SO and S/E proteins are shown in Figs. 1 and 2, after the protein transfer and immunoblotting, the results of the immunoblotting of serum samples collected from naturally and experimentally infected *F. gigantica* cattle are shown in Figs. 3 and 4. A representative blot is shown for each antigen preparation.

Several protein bands were identified in SO and E/S products of *F. gigantica* by the sera of both experimentally and naturally infected cattle. Proteins of 27-30 Kda were detected in SO and E/S products of the parasite by antibodies from naturally infected cattle (Fig 3). The same proteins were also detected in both antigen preparations eight

weeks post-infection (P.I.) by sera of experimentally infected cattle (Fig 4).Proteins of 50 Kda and 66 Kda were also faintly recognized in E/S products 8 weeks P.I. in experimentally infected cattle (Fig. 4).

Additionally, Protein bands of 40-60 Kda were detected after 5 weeks PI whereas protein of 100 and 120 Kda were recognized eight weeks PI.

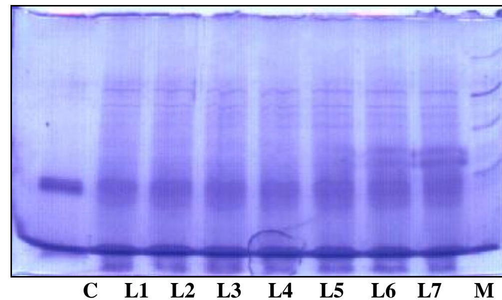

Fig 1: SDS-PAGE electrophoresis of S/E protein products of *F.gigantica* (L1-L7), purified cycteine proteinase (Lane C) and molecular marker (Lane M).

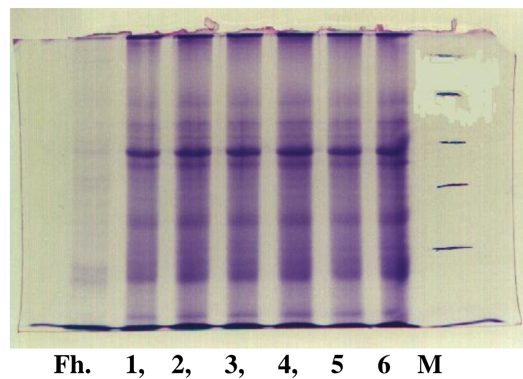

Fig 2: SDS-PAGE electrophoresis of SO protein of *F.gigantica* (Lane1-Lane 6), *F. hepatica* (Lane Fh) and molecular marker (Lane M).

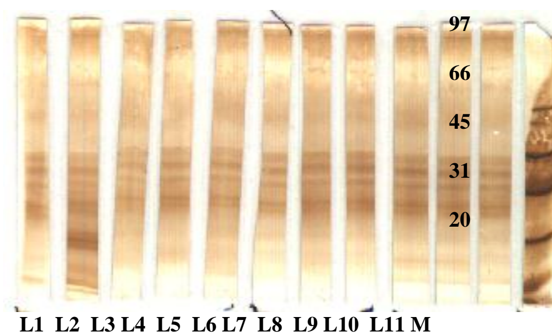

Fig. 3: **Immunoblotting of E/S products against immune sera from *F.gigentica* experimentally infected cattle (L8-L11), M= molecular marker.**

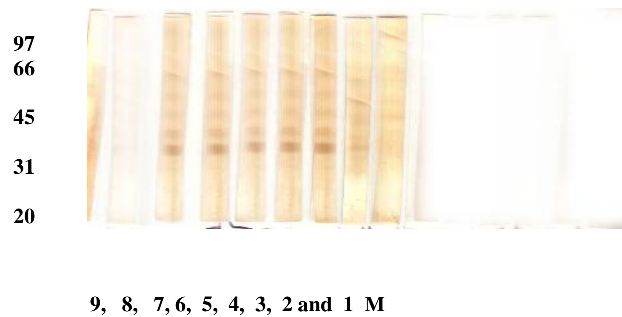

Fig. 4: Immunoblotting of SO protein products against immune sera of F. gigantica naturally infected cattle.

Discussion

In the present study, the antigenic recognition of protein composition of E/S and SO products of *F. gigantica* was investigated by SDS-PAGE and Western immunoblotting techniques. Separation by SDS-PAGE allowed the determination of the relative molecular weights and demonstrated the abundance of the components present in these parasite preparations. E/S and SO products of *F. gigantica* were probed with serum from both *F. gigantica* naturally and experimentally infected cattle by Western blot, in order to identify immunoreactive antigenic polypeptides.

Immunoreactive antigenic polypeptides of molecular weight between 27 to 30 KDa were identified in both E/S and SO preparations in both naturally and experimentally *F. gigantica* infected cattle four weeks P.I. The intensity of the reaction in experimentally infected cattle increased steadily with increase in the duration of the infection. Despite the fact that less protein bands were detected by western blots of E/S products compared to that in SO preparation, the proteins bands had the same intensity in the two products. Several investigators have similarly identified antigenic components in E/S and SO products of *F. hepatica* within the range of 20 to 30 KDa in different animal species including man (Coles and Rubano, 1988; Santiago and Hillyer, 1988; Ruiz-Navarrete *et al*., 1993; Chauvin *et al*, 1995).The present results, confirm previous reports (ref.) on the presence of antigenic components of almost similar molecular weights in adult *F. gigantica* E/S and SO products.

In conclusion, the major antigenic proteins in both E/S and SO products of (adult or juvenile) *F. gigantica* were present within the range of 27 to 30 Kda. However, the E/S products are apparently more immunogenic. This is probably because they contain highly immunogenic proteins such as cysteine L-proteinase and glutathione S tranferase (ref.) which also were identified in this study. These proteinases may have the potential of being used for early diagnosis of fasciolosis and they could further be an effective vaccinal candidate.

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