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SNAIL DERIVED ANTIGENS USEFUL IN DIAGNOSIS OF FASCIOLIASIS

(With 3 Figures)

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مولدات ضد من القوقع لها أهمية فى تشخيص الإصابة بالديدان الكبدية

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فى هذه الدراسة تم تقييم وتصنيف مولدى الضد لقوقع ليمنيا (القدمى والكبدى البنكرياسى) فى التشـخيص المناعى المتخصص للاصابة بالديدان الكبدية باستخدام اختبارى اليزا والويسترن بلـوت. أظهر اختبار اليزا نجاح مولدى الضد لقوقع ليمنيا فى الكشف عن الأجسام المضادة للديـدان الكـبدية العملاقـة (IgG) فى حيوانات مصابة حقليا بالمرض. الأ أن مولد الضد الكـبدى البنكرياسى لقوقع ليمنيا أظهر حساسية أعلى من مولد الضد القدمى فى الكشف عن الأجسـام المضـادة. كمـا أسفر التحليل الكهربى فى البولى أكريلاميد جل عن وجود حزم بروتينية مشتركة بين مولد الضد الاخراجى الافرازى للديدان الكبدية العملاقة ومولدى الضد لقوقـع ليمنيا. ومن ثم أمتدت هذه الدراسة لتقييم تلك الحزم البروتينية المشتركة وخاصة فى مولـد الضد الكبدى البنكرياسى لقوقع ليمنيا فى الكشف عن الأجسام المضادة للديدان الكبدية فـى الحيوانات المصابة حقليا باستخدام الويستيرن بلوت. حيث أوضح هذا الاختبار انه من بين العديد من البروتينات لمولد الضد الكبدى البنكرياسى لقوقع ليمنيا التى أظهرت تفاعلات اجابـية مـع أمصال حيوانات مصابة بالديدان الكبدية، كان البروتين ذو الوزن الجزئى ٦٤ كيلو دالتون مشترك مع مولد الضد الاخراجى الافرازى للديدان الكبدية العملاقة ولم يكن له وجود فى مولد الضد القدمى لقوقع ليمنيا. هذا البروتين ربما يكون المسؤل عن حساسية مولد الضـد الكـبدى البنكرياسى لكى تكون أعلى من حساسية مولد الضد القدمى فى الكشف عن الأجسام المضادة. قدم هذا البحث اتجاها جديدا فى التشخيص المناعى لمرض الديدان الكبدية باسـتخدام النسيج الكبدى البنكرياسى لقوقع ليمنيا، حيث يعد كمصدر غنى لمولد الضد علاوة علـى سهولة الحصول عليه وتكلفته المنخفضة بالمقارنة بمولدات الضد للديدان الكبدية، مثل الاخراجى الافرازى والخام والبويضى.

SUMMARY

L. cailliaudi feet and hepatopancreases antigens which would be of use as a specific immunodiagnostic test for fascioliasis were evaluated and characterized using ELISA and Western blot techniques. ELISA tests

demonstrated the successful detection of *F. gigantica* IgG antibodies in naturally infected animals using both antigens. But, *L. cailliaudi* hepatopancreases antigen showed higher sensitivity in antibody detection than *L. cailliaudi* feet. SDS-PAGE provided evidence of shared antigens between *F. gigantica* ES, *L. cailliaudi* feet and *L. cailliaudi* hepatopancreases antigens. Those observations were extended by demonstrating the value of the antigenic epitopes of *L. cailliaudi* hepatopancreases in detection of anti-*Fasciola* antibodies in naturally infected animals using western blot technique. Where, from several polypeptides showed specific reactivity toward *F. gigantica* infected animal sera, the 64 KDa polypeptide was common with *F. gigantica* ES antigen and was not detected in *L. cailliaudi* feet antigen. This polypeptide might be reinforced the sensitivity of *L. cailliaudi* hepatopancreases antigen to be higher than that of *L. cailliaudi* feet antigen. These results suggested that *L. cailliaudi* hepatopancreases constituted a rich source of antigen for serodiagnostic studies of *F. gigantica*. Besides, it was easy to obtain and with a low cost compared to other *Fasciola* antigens as ES, crude worm and egg.

***Key words**: Lymnaea cailliaudi, Fasciola gigantica, antigen, ELISA, Western blot.*

INTRODUCTION

Several studies had dealt with the concept of common antigens between trematodes and their intermediate hosts (Chaçon *et al.*, 2002 and El-Bahy and Shalaby, 2004) and the possibility of using these antigens for serodiagnosis of their target parasites (Alarcon de Noya *et al.*, 1989). Moreover, Kagan (1955) demonstrated that sera of rabbits immunized with extracts of the hepatopancreases of infected *Biomphalaria glabrata* or *Lymnaea* spp. snails were positive in the Cercarien-Hullen Reaction. Capron *et al.* (1965) identified 4 antigens in *Schistosoma mansoni* adults; reacted specifically with an anti-*Biomphalaria glabrata*- serum. They suggested that these reactions might be due to the adaptation of the parasite with its intermediate host. They were subsequently able to show that humans with schistosomiasis developed 3 precipitins reactive with *B. glabrata* extracts using immunoelectrophoresis (Capron *et al.*, 1969). Furthermore, Capron *et al.* (1968) were able to demonstrate common antigenic communities between other trematodes (S. *haematobium, S. japonicum, Paragonimus*

westermani) and their intermediate hosts as well as antigenic communities within intermediate hosts. Rasmussen *et al.* (1985) carried out a study to isolate, purify and characterize a shared antigen between S. *mansoni, Fasciola hepatica* and *B. glabrata*; which would be of use as a specific immunodiagnostic tests for schistosomiasis. The results showed a limited value for this shared antigen for the specific immunodiagnosis of schistosomiasis, but did suggest a possible potential as a general screening tool for detecting trematode infections. Alarcon de Noya *et al.* (1989) cleared that serum from *Schistosoma* infected persons reacted versus crude *B. glabrata* antigen by ELISA; with sensitivity rate reached up to 100%.

In a closely related study, El-Bahy and Shalaby (2004) demonstrated the presence of a similarity in antigenic composition between snails and their parasites using SDS-PAGE and Western blot techniques. The fact that facilitated development of the parasite in its specific snail host. This antigenic similarity had not been recorded with foreign parasite. In the present study, the serodiagnostic potential of *Lymnaea cailliaudi* antigens (intermediate host of *Fasciola gigantica*) in diagnosis of natural *F. gigantica* infection in animals at different levels of infection was evaluated using ELISA and Western blot techniques.

MATERIALS and METHODS

Preparation of antigens

Snail antigens

Field – collected *L. cailliaudi* snails, intermediate host of *F. gigantica*, were identified according to Brown (1994) and reared in the laboratory for production of laboratory-bred snails according to El-Bahy (1984). They were used for antigen preparation according to Khalil *et al.* (1985) and El-Bahy and Shalaby (2004). Feet and hepatopancreases of laboratory bred mature non-infected snails were dissected. The collected tissues were homogenized in an equal amount of 0.01 M phosphate buffered saline, PH 7.4 (PBS) and sonicated for 5 minutes under 150 watt interrupted pulse cycle using a sonifier cell disrupter. Then, they were centrifuged at 5000 rpm for one hour at 4 °C. The supernatant was dialyzed in 6 – 8 KDa dialysis tubes overnight at 4 °C against 4 M urea buffer. Thereafter, they were concentrated by absorption against polyvinyl pylorridone. The protein content was measured by the method of Lowry *et al.* (1951) and stored at – 70 °C until used.

***F. gigantica* antigens**

***F. gigantica* excretory-secretory antigen (ES antigen)**

F. gigantica ES antigen was prepared from living flukes collected from fresh condemned buffaloes' livers at Cairo abattoir according to Rivera-Marrero *et al.* (1988). After 3 hours incubation in 0.01 M PBS, PH 7.4 at 37 °C (40 worms/100 ml PBS), the supernatant was separated after centrifugation at 10000 rpm at 4 °C for one hour. The protein content was determined by the method of Lowry *et al.* (1951). The antigen was aliquoted and stored at – 70 °C until used.

***F. gigantica* crude worm antigen**

F. gigantica crude worm antigen was prepared according to Shalaby (1998) from the anterior parts of fresh extracted adult *F. gigantica* worms collected from fresh condemned buffaloes' livers. They were washed repeatedly in 0.01 M PBS, PH 7.4. and homogenized at 6000 rpm for 20 minutes, and then subjected to high-speed centrifugation (10000 rpm) for one hour at 4 °C. The supernatant was separated as crude antigen after the protein content had been measured as above and stored at – 70 °C until used.

***F. gigantica* egg antigen**

F. gigantica egg antigen was prepared according to El-Bahy *et al.* (1992). The eggs were collected from the bile of infected slaughtered animals by sedimentation methods using several changes of tap water. The eggs were washed 3 times with distilled water containing penicillin G (500 IU/ml) and gentamycine (50 mg/ml). Then, the collected eggs were sonicated with 0.01 M PBS, PH 7.4 for 10 minutes and subjected to high-speed centrifugation (10000 rpm) for one hour at 4 °C. The supernatant was separated as egg antigen after the protein content had been measured as above and stored at – 70 °C until used.

Preparation of sera

Animal sera

A total of 15 selected samples of known parasitic infected history were tested. The collected sera were arranged into two groups. The first group was 10 serum samples of buffaloes harboring *Fasciola* eggs only in their feces [5 of low egg/gram of feces (EPG) (1-4 egg) and 5 of high EPG (5 eggs or more) according to Malone *et al.* (1987)]. The second group was 5 serum samples collected from young buffalo calves as non-infected control animals.

Reference rabbit hyper-immune sera

Rabbit hyper-immune sera were raised against four antigens (*L. cailliaudi* feet, *L. cailliaudi* hepatopancreases, *F. gigantica* crude

worm and *F. gigantica* ES) as described by Langley and Hillyer (1989) via initial subcutaneous injection in an equal volume of Freund's complete adjuvant and three consecutive intramuscularly injections in an equal volumes of Freund's incomplete adjuvant during 60 days. The level of specific antibodies in sera of immunized rabbits was evaluated before slaughter. Rabbits were bled before immunization as negative control sera.

Enzyme-linked immunosorbent assay (ELISA)

Antibody levels in the previous sera were measured by an ELISA using the tested antigens according to the procedure of Oldham (1983). Checkerboard titrations were performed in order to establish the optimum concentration for the tests. The assay was performed in 96-well-flat bottom microtiter plates; which were coated by overnight incubation at 4 °C with 100 µl aliquots(per well) of test antigen (4 µg per ml of 0.1M sodium carbonate buffer, PH 9.6). Sera from *F. gigantica* infected animals and non-infected control animals as well as HIS raised against *F. gigantica* crude worm and ES antigens were serially diluted (1:50 to 1:1600). The sera were added in duplicate wells (100 µl per well) and incubated at 37 °C for 1 hour. Peroxidase – conjugated protein A (Sigma) was diluted 1000-fold, 100µl aliquots added per well and incubated as described. Reagents were diluted in 0.01M PBS, PH 7.4 containing 0.05% Tween 20 (Sigma) (PBS-T). Wells were washed between incubations with PBS-T. The absorbance of the peroxidase reaction (determined with orthophenylenediamine 340 µg/ml citrate/phosphate buffer, PH 5.0 with 0.03% hydrogen peroxidase solution) was measured at 490 nm using ELISA reader.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) and Western blot techniques

Protein fractions of each tested snail antigens (*L. cailliaudi* feet and *L. cailliaudi* hepatopancreases) in comparison with *F. gigantica* ES antigen were demonstrated using 12% SDS-PAGE (100ug/lane) according to Laemmli (1970) with the aid of low molecular weight standard (Pharmacia Biotech). The fractionated *L. cailliaudi* feet and hepatopancreases antigens were transferred onto nitrocellulose sheet for western blot technique according to Towbin *et al.* (1979). The nitrocellulose strips blotted with snail antigens were tested in group of three as in Fig. 3:

- The first strip was used to test the recognition of the original molecules of snail antigen by homologous immune sera.

- The second one was allowed to react with *F. gigantica* infected animal serum.
- The third one was allowed to react with non-infected control animal serum.

The molecular weight of specific and non-specific polypeptides was determined using low molecular weight standard curve as described by the producer (Pharmacia).

RESULTS

Antibody levels in *F. gigantica* infected animal sera; either with low or high EPG and rabbit HIS raised against *F. gigantica* ES and crude worm antigens at serial dilution (1:50 to 1:1600), were measured by an ELISA using the tested antigens. As could be observed in Fig. 1; results of the ELISA in *F. gigantica* infected animal sera with low EPG, revealed that the mean OD values increased with increasing serum dilution till reached the highest levels at 1:800 dilution with all tested antigens. The only exception was for *F. gigantica* egg antigen with which the highest level of the mean OD value was at 1:400 serum dilution. Thereafter, the mean OD values with all tested antigens decreased with increasing serum dilution to 1:1600. *L. cailliaudi* feet and hepatopancreases antigens demonstrated a greater reactivity in infected animal sera than in non-infected sera. However, at high serum dilution (1:800), *L. cailliaudi* hepatopancreases antigen showed higher mean OD value than *L. cailliaudi* feet antigen; 0.792 and 0.497, respectively. At the same time, it was very close to that recorded with *F. gigantica* ES (0.679) and crude worm antigens (0.735) and higher than that recorded with *F.gigantica* egg antigen (0.464).

On the other hand, in *F. gigantica* infected animal sera with high EPG, the mean OD values decreased with increasing serum dilution till reaching negative values at dilution 1:1600 with all tested antigens, except for *L. cailliaudi* feet antigen; with which the mean OD value reached the negative level at dilution 1:800. Thus, *L. cailliaudi* hepatopancreases antigen could detect *F. gigantica* IgG antibodies at high serum dilution (1:800) while, at this dilution, *L. cailliaudi* feet antigen failed to detect antibodies. The mean OD value recorded in infected animal sera at this high dilution using *L. cailliaudi* hepatopancreases antigen was 0.145. It was very close to that recorded with *F. gigantica* ES antigen (0.135) and lower than that recorded with

F. gigantica crude worm antigen (0.238). While, it was still higher than that recorded with *F. gigantica* egg antigen (0.117).

L. cailliaudi hepatopancreases antigen showed higher cross-reactive binding activities toward *F. gigantica* ES and crude worm HIS than *L. cailliaudi* feet. *F. gigantica* ES and crude worm antigens, as it was expected, showed strong cross-reactive binding activities with heterologous HIS; that was still present even with high serum dilution. While, *F. gigantica* egg antigen showed strong binding activity with *F.gigantica* crude worm HIS but not with *F. gigantica* ES HIS.

From these observations, it was obvious that *L. cailliaudi* hepatopancreases antigen appeared to be more sensitive in detection of *F. gigantica* IgG antibodies than *L. cailliaudi* feet and showed mean OD values in *F. gigantica* infected animal sera very close to that recorded with *F. gigantica* ES antigen.

Analysis by SDS-PAGE of *L. cailliaudi* feet and hepatopancreases in comparison with *F. gigantica* ES antigen revealed at least 10, 13 and 14 polypeptides in each antigen, respectively, as shown in Fig.2. These polypeptides molecular weight ranged from 16-160 KDa. The protein bands at molecular weight of about 70, 54, 42, 34, 28 and 22 KDa were common among the three tested antigens. Notably, one protein band more in *L. cailliaudi* hepatopancreases antigen at molecular weight of 64 KDa shared with *F. gigantica* ES antigen.

Western blot technique was performed to find out the antigenically active components in *L. cailliaudi* feet and hepatopancreases; that might be responsible for immunoreactivity with *F. gigantica* infected animal sera among the several polypeptides of each antigen. The adopted Western blot technique (Fig.3) revealed that *L. cailliaudi* feet HIS reacted specifically with nine polypeptides present in its own antigen (*L. cailliaudi* feet) at molecular weight of 94, 85, 75, 70, 66, 57, 54, 51, and 45 KDa (Fig.3 Lane A). From these polypeptides, only three at molecular weight of 85, 70 and 54 KDa were recognized by *F. gigantica* infected sera (Fig.3 Lane B). While, no polypeptides were identified by non-infected control animal sera (Fig.3 Lane C). Therefore, the three polypeptides of 85, 70 and 54 KDa were considered to be specific immunoreactive shared polypeptides between *F. gigantica* and *L. cailliaudi* feet.

Concerning *L. cailliaudi* hepatopancreases antigen, eight polypeptides at molecular weight of 97-85, 70, 64, 54, 45, 42, 40 and 38 KDa were recognized by its homologous HIS (Fig.3 Lane D). Six of these polypeptides of 97-85, 70, 64, 54, 45 and 42 KDa were identified

by *F. gigantica* infected animal sera (Fig.3 Lane E). On the other han, there was a non-specific reaction at 97-85 KDa on using non-infected control animal sera (Fig.3 Lane F). Thus the polypeptides of 70, 64, 54, 45 and 42 KDa were considered to be specific immunoreactive shared polypeptides between *F. gigantica* and *L. cailliaudi* hepatopancreases.

DISCUSSION

The use of snail intermediate host antigens for immunodiagnosis of schistosomiasis had been summarized by Kagan and Pellegrino (1961). However, there were no reports dealing with the use of *L. cailliaudi* antigens as a possible alternative in serodiagnosis of fascioliasis. The original goal of this study was to evaluate and characterize *L. cailliaudi* feet and hepatopancreases antigens which would be of use as a specific immunodiagnostic test for fascioliasis.

An important fact of the present study was the successful detection of *F. gigantica* IgG antibodies by using *L. cailliaudi* snail antigens (feet and hepatopancreases). Where, they demonstrated a greater reactivity in *F. gigantica* infected animal sera than in non-infected animal sera. But, *L. cailliaudi* hepatopancreases antigen showed higher sensitivity in antibody detection than *L. cailliaudi* feet antigen. This fact that was proved via ELISA by obtaining mean OD values, at different levels of infection, in *F. gigantica* infected animal sera with *L. cailliaudi* hepatopancreases antigen very close to that recorded with *F. gigantica* ES antigen that had been previously described as the best candidate for immunodiagnosis of fascioliasis in cattle and sheep (Rivera-Marrero *et al.*, 1988).

The ELISA results showed failure of *L. cailliaudi* feet antigen in detection of anti-*Fasciola* antibodies, in group of animals shedding high EPG, at high serum dilution (1:800). This might be attributed to presence of low antibody titers that could not be detectable by *L. cailliaudi* feet antigen in this group of animals associated with chronic infection (El-Bahy, 2002).

The relevance of the *L. cailliaudi* antigens to the host-parasite relationship was supported by the presence of specific circulating antibody in infected, but not normal animal (Rasmussen *et al.*, 1985). This suggested that the host naturally recognized and responded to the antigens as well as a possible contribution of those antibodies in protecting animals against infection by the parasite. Indeed, Mohamed (1993) reported that *L. cailliaudi* hepatopancreases antigen was capable

of inducing significant protection to a challenge infection of *F. gigantica* in rabbits. Additionally, Chieffi *et al.* (1982) reported higher titers of anti-*B. glabrata* antibodies in the serum of humans and mice infected with *S. mansoni* than in controls when tested by passive hemagglutination.

Moreover, the ELISA results demonstrated the higher cross-reactive binding activities of *L. cailliaudi* hepatopancreases antigen toward *F. gigantica* ES and crude worm HIS than *L. cailliaudi* feet antigen. These results concurred with those obtained by Mohamed (1993) who mentioned that both of *L. cailliaudi* hepatopancreases and adult *F. gigantica* ES antigens produced a precipitin band against *F. gigantica* ES HIS using agar gel diffusion test.

The protein banding patterns on SDS-PAGE of *L. cailliaudi* feet and hepatopancreases as well as *F. gigantica* ES antigens revealed shared bands among the three tested antigens. These protein bands were at molecular weight of about 70, 54, 42, 34, 28 and 22 KDa. This appeared to be in line with the findings of Chacon *et al.* (2002) that confirmed the presence of common antigens between *S, mansoni* and its vector, *B. glabrata.*

By western blot technique, it was demonstrated that in *L. cailliaudi* feet antigen, there were three of the nine polypeptides identified by homologous HIS showed specific positive reactivity with *F. gigantica* infected animal sera. These polypeptides were at molecular weight of 85, 70 and 54 KDa. Meanwhile, there were five of the eight polypeptides in *L. cailliaudi* hepatopancreases antigen identified by homologous HIS at molecular weight of 70, 64, 54, 45 and 42 KDa, showed specific positive reactivity. In that sense, Rivera-Marrero and Hillyer (1988) demonstrated that there were four peptides in *B. glabrata* soluble hepatopancreases antigen reacted specifically with serum of mice infected with *S. mansoni* for 9 weeks. Those peptides were at molecular weight of 66, 50, 48 and 32 KDa. They suggested that four specific shared antigens were peptides important in a primary infection with *S. mansoni.*

As mentioned earlier, in SDS-PAGE analysis, the 64 KDa polypeptide in *L. cailliaudi* hepatopancreases antigen; that showed specific positive reactivity toward *F. gigantica* infected animal sera, was common with *F. gigantica* ES antigen and was not detected in *L. cailliaudi* feet antigen. This polypeptide was of special interest in view of the fact Mousa (1992) had shown that the 63 KDa fraction of *F. gigantica* ES antigen was found to be sensitive and specific for diagnosis

of acute fascioliasis. Therefore, this polypeptide might be reinforced the sensitivity of *L. cailliaudi* hepatopancreases antigen to be higher than that of *L. cailliaudi* feet antigen in detection of anti-*Fasciola* antibodies as observed in ELISA test.

The present study demonstrated the higher sensitivity of *L. cailliaudi* hepatopancreases antigen than *L. cailliaudi* feet antigen in detection of *F. gigantica* IgG antibodies. It provided evidence of shared antigens between *F.gigantica* and *L. cailliaudi*, and extended those observations by demonstrating the value of the antigenic epitopes of *L. cailliaudi* hepatopancreases in detection of anti-*Fasciola* antibodies in naturally infected animals. Apparently; from this investigation we could notice that the *L. cailliaudi* hepatopancreases constituted a rich source of antigen for serodiagnostic studies for *F. gigantica*. Besides, they were easy to obtain, with a high recovery of proteins and low cost, compared to other *Fasciola* antigens; like ES, crude worm and egg.

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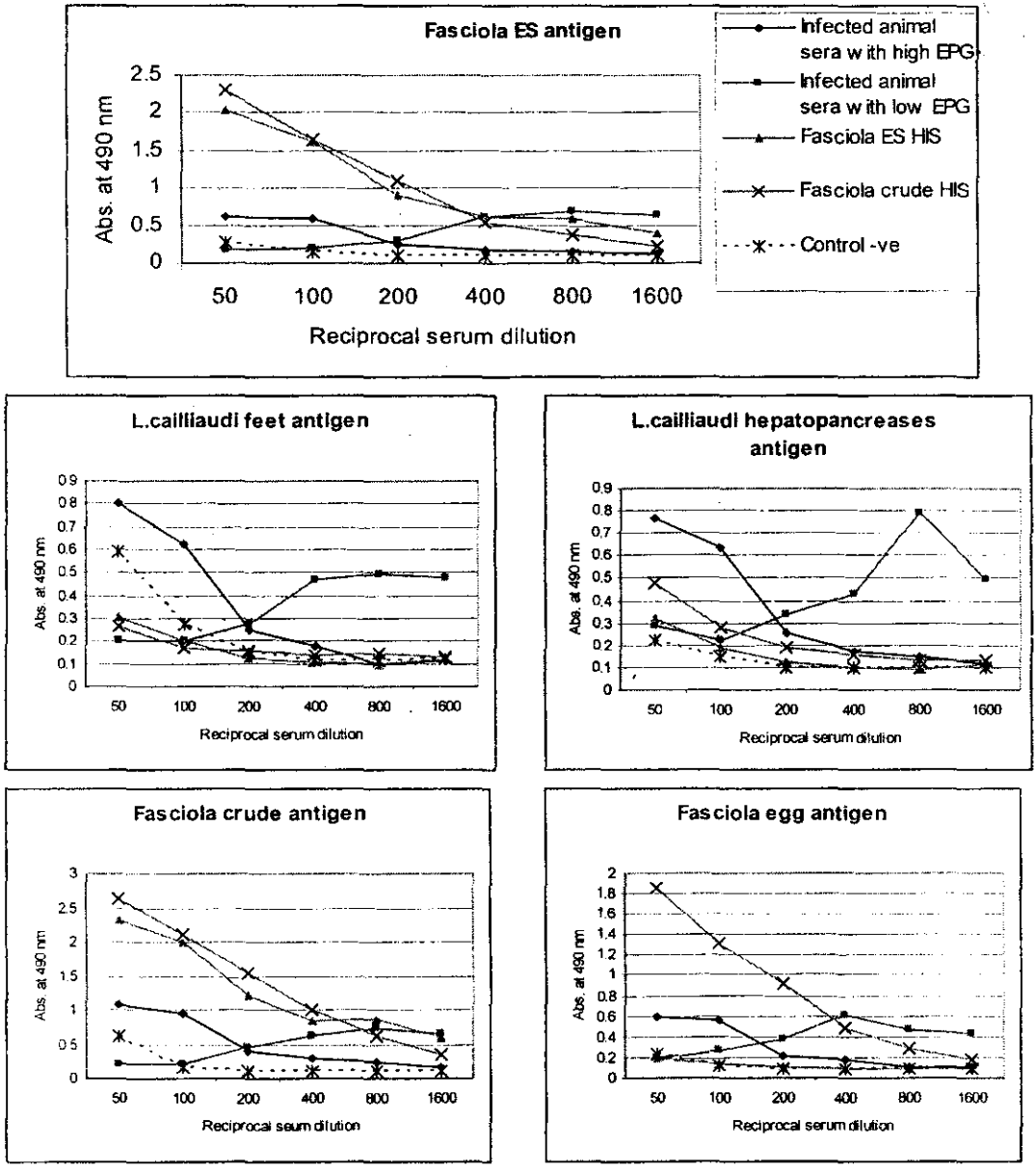

Fig. 1: ELISA profile demonstrating reactivity of *F. gigantica* infected animal sera associated with high & low EPG, *F. gigantica* ES and crude HIS and non-infected animal sera (control –ve) toward the tested antigens.

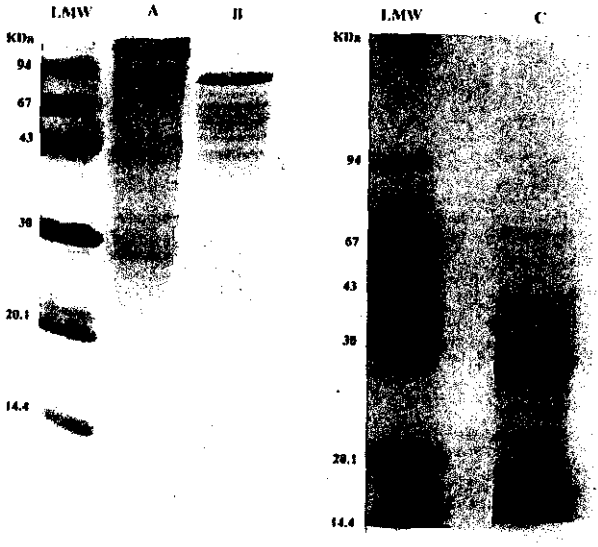

Fig. 2: SDS-PAGE of *L. cailliaudi* feet (Lane A), *L. cailliaudi* hepatopancreases (Lane B) and *F. gigantica* ES (Lane C) antigens.

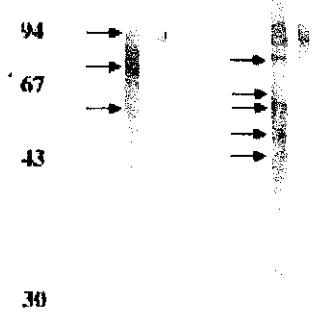

Fig. 3: Recognition of different snail antigens by *F. gigantica* infected animal sera using western blot.

I. *L. cailliaudi* feet antigen.

II. *L. cailliaudi* hepatopancreases antigen.

Lane A. with *L. cailliaudi* feet HIS.

Lane D. with *L. cailliaudi* hepatopanceases HIS.

Lane B & E. with *F. gigantica* infected animal sera.

Lane C & F. with non-infected animal sera.

LMW. Low Molecular Weight marker stained with Ponceau s solution.