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CHARACTERIZATION OF ANTIGENIC PROPERTY OF FASCIOLA HEPATICA AND FASCIOLA GIGANTICA

BY

*SAHLAB A.A.M., and **ABDEL-AAL, A.A.

* Animal Health Research Institute . Devices and Biotechnology unit.
** Department of Parasitology Faculty of Vet. Med. Suez canal Univ.

SUMMARY

Differential molecular studies was performed between *Fasciola hepatica* and *Fasciola gigantica*. The total protein content of whole *F. hepatica* antigen (85mg/gm) seemed to be double value of *F. gigantica* (42mg/gm) while the excretory-secretory product of *F. hepatica* and *F. gigantica* was 60 and 52mg/gm respectively. SDS-PAGE of both whole and excretory-secretory antigen of *F. hepatica* as well as *F. gigantica* showed common bands at 14.5kd. and 28kd. and each one had characteristic bands clustered in different molecular weights. The western blot showed cross reactive immune bands at 14.5kd. and 28 kd. while 47kd. was specific to *F. hepatica* but the bands located at 57kd, 69kd, 88kd were specific to *F. gigantica*. The western blot was more sensitive and can predict infected case than abattoir post mortem examination. The work can be used to determine the early incidence of fascioliasis including Fasciola species.

INTRODUCTION

Both species of *Fasciola hepatica* and *Fasciola gigantica* are common parasites of domestic livestock, cause severe disease and important economic losses in the animal husbandry industry. The clinical history and experience of liver flukes in endemic areas in addition to detection of Fasciola eggs in feacal samples of cattle is the most reliable diagnostic method for fascioliasis. However, it is characterized by lack of sensitivity for detecting the helminthoses because eggs are found only after eight weeks post infection as well as low-level of parasitic infestation de Leon, et al., (1981), Hillyer, et al., (1985), and Fagbemi and Guobadia (1995). In Egypt the incidence average of the disease in cattle at Sharkia abattoirs was 30.2%. EL-Atabany (1986), while Salem, et al., (1990) recorded the infestation rate depend on coprological diagnosis, among cattle, buffaloes and sheep to be 26.3%, 23.5% and 39.3% respectively. EL-Bahy (1997) mentioned that the results of examination of faecal samples collected from

humans and their surrounding animals in six selected places in Kafr -EL-Sheikh governorate for the presence of Fasciola eggs were as follows: 14.5% of buffaloes, 26.6% of sheep, 12.28% of donkeys and 6.02% of human. The diagnosis of parasitic infection is nowadays directed towards the immunological detection of parasite or its circulating excre-torysecretory antigens in the host body fluids (Chandrashekar, et al., 1990, Maizels, et al., 1990, Zheng, et al., 1990, and Wonsit, et al., 1992). In Egypt, no data are available on the diagnosis of fascioliasis using molecular technique. The aim of this work is to identify the immunoreactive proteins of a secretory-excretory preparation of *F. hepatica* and *F. gigantica* affecting cattles slaughtered in local El-Basatein and Ismailia abattoirs with reference to experimentally immunized rabbit with whole protein of worms. This work will detect prepatent or occult infections which are undetected by usual parasitological tests.

MATERIALS AND METHODS

Preparation of excretory-secretory F. hepatica and F. gigantica antigens.

Fasciola hepatica and F. gigantica (Fig 6) were obtained and identified as described pre-viously (Soulsby, 1982) and the excretory-secretory antigens was prepared according to Fagbemi, et al., (1997), with some modification briefly, the living F. hepatica and F. gi-gantica worms were isolated from the bile ducts of slaughtered and seemed to be mono infected cases at local El-Basatein and Ismailia abattoirs. The adult worms were washed several times in cold PBS 4oc to remove all blood and bile traces then weighed separately and incubated 3 hours at room temperature in PBS containing 10mM leupeptin (5 ml/worm). Fifty mM tris pH8, 5mM EDTA, 5mM iodo acetamide, 0.1mM TLCK, and 44mM PMSF were added to the medium, then centrifuged at 60,000 xg and the supernatant was concentrated 20 fold by means of freez-drying machine (BETA-1 Germany) at -80°C.

Preparation of whole F. hepatica and F. gigantica antigens.

The *F. hepatica* and *F. gigantica* worms were isolated from the bile ducts of slaughtered and suspected mono infected cases as mentioned previously. The adult worms were washed 6x in cold PBS then weighed and homogenized in 5 ml of antiprotease buffer (50 mM tris pH 8, 5 mM EDTA, 5mM iodo acetamide, 0.1mM TLCK, 44mM PMSF and 10mM leupeptin). The supernatant was obtained by centrifugation at 20,000xg at 4°C for 30 minutes to remove the particulate material and eggs then concentrated 10 fold by means of freez-drying machine. The protein concentration of whole and excretory-secretory antigens was determined separately according to Bradford (1976) and stored as 5 mg/ml at -20°C till use.

Blood samples and sera .

One hundred serum samples were collected from slaughtered cattle. The mono infected cases with *F. hepatica* or *F. gigantica* were selected visually based on the size of worm. The collected sera were selected from clinically infected and

negative cases according to the post mortem examination and used as positive and negative control. All sera were stored at -20°C till use.

Rabbit immunization with whole-worm antigen .

twelve males of New Zeeland white rabbits of 6 months age each were obtained from clean hygienic farm fed on dry concentrated ration. They were divided into three groups. The first group was injected subcutaneously as described previously by Fagbemi and Obarisiagbon (1991) with 100 mg of whole-worm antigen of *F. hepatica* in Freund's complete adjuvant in the ratio 1:1. The second group was injected with whole-worm antigen of *F. gigantica* as in previously mentioned step. A booster dose of 50 mg antigen in Freund's incom-plete adjuvant was given on day 14. The second and third booster doses were given on days 21 and 28. The post serum samples were collected 4 days after the last immunization. The third group was left as control.

Analysis of *F. hepatica* and *F. gigantica* proteins by SDS-PAGE and immunoblotting.

The proteins of excretory-secretory as well as whole protein extract of *F. hepatica* and *F. gigantica* antigens were separated and optimized in 5-15 gradient (SDS-PAGE) at constant 40 mA for 3 hours, then transferred to a 0.45mm nitrocellulose filters using trans-blot electrophoretic transfer cell (BIO-RAD) according to Towbin and Gordon (1984) and stored in dry place till use. The excretory-secretory loaded filter was blocked in 5% milk /PBS/tween-20 solution (pH 7.4) for one hour (Johnson et al., 1984). The infected and control sera were diluted 1/300 and probed on nitrocellulose separated filters for one hour followed by secondary antispecies sera (antirabbit in case of primary experimentally control rabbit anti-Fasciola sera or antibovin in case of cattle sera) labeled with Horseradish Peroxidase (SIGMA) in a concentration of 1/2000 at room temperature. The blots were developed with 3,3-diaminobenzidine enhanced by cobalt chloride as detection system (Guobadia and Fagbemi 1997). The stained protein profile in the gels as well as the detected immune reactive bands on nitrocellulose filters were densitometry analyzed in comparing with standard molecular weight rainbow markers ranged from 14.3kd. to 200kd. (Amersham life science Co.) to estimate the polypeptides profile fingerprint of each Fasciola species and detect the major immune reactive bands of each.

RESULTS

protein evaluation of the whole worm antigen as well as excretory-secretory antigen.

The main protein concentration of whole worm antigen of *F. hepatica* was determined as 85mg/gram of worm mass while it showed 42 mg in case of *F. gigantica*. The main protein concentration of excretory-secretory preparation content of *F. hepatica* was determined as 60 mg/gram of worm mass while it showed 52mg in case of *F. gigantica*.

SDS-PAGE analysis.

The optimum loading concentration of the whole worm antigen as well as excretory-secretory antigen (Fig.1, 2, 3 and 4) of both species was 75ug/well which electrophoretically separated on 5-15 gradient SDS-PAGE. The discending densitometry of first sixth major commassei stained banding patterns for whole worm antigen of *F. hepatica* were appeared approximately at 27.75kd. (represented 10.3% of total separated sample) followed by 14.1kd. (7.1%), 41.4 kd.% (5.7%), 47.2kd. (4.7%), 33.9kd.(4.3%), 69.5kd.(3.3%) while other clusters of bands fluctuated between 11.9kd.-238.4kd. The discending arrangements of the first sixth major banding patterns of *F. gigantica* were 28kd. (representing 10.9% of total separated sample) 14.4kd. (10.4%), 34.5kd. (5%), 120kd. (3.6%), 48.5kd. (2.9%), 52.3kd. (2.5%) while other clusters of bands fluctuated between 10.7kd.-231.1kd. The predominant common bands for both parasitic species were clustered nearly at 14kd., 28kd., 34kd. (Fig. 1). The discending densitometry of major commassei stained banding patterns for excretory-secretory antigen of *F. hepatica* were clustered at 14.1kd., 27.5kd. and 46.2kd. while for *F. gigantica* they were clustered at 14.6kd., 27.8kd., 56.3kd., 68kd., 87.2kd. (Fig.2).

Enzyme-Linked Immuno Electrotransfer Blot analysis (Western Immunoblot).

Sera obtained from the experimentally immunized rabbit with whole *F. hepatica* antigen reacted with separated excretory-secretory antigen of the same parasite loaded on the nitrocel-lulose paper at 14.5kd., 28kd., 47kd. The sera obtained from the experimentally immunized rabbit with whole *F. gigantica* antigen reacted with separated excretory-secretory antigen of *F. gigantica* and showed bands approximately at 14.5kd., 28kd., 57kd., 69kd. and 88kd. The sera collected from infected cattle with *F. hepatica* reacted with excretory-secretory antigen of the same parasite intensively at 14.5kd., 28 kd. and faint reaction was observed at 47kd. (Fig. 5). All collected sera from infected cattle with *F. gigantica* reacted with the same parasitic excretory-secretory antigen on nitrocellulose paper mainly at 14.5kd, 28kd. and faint reaction was observed at 57kd., 69kd., 88kd. Sex cattles were positive to fascioliasis in post mortem examination representing infection rate of 6% and their sera reacted commonly with 14.5kd, 28kd. of excretory-secretory antigen of *F. hepatica* and *F. gigantica* antigens. Only two cases had im-munoreactive bands at 14.5kd., 28kd., 47kd, and 69kd. while their post mortem examination revealed only the presence of *F. hepatica*.

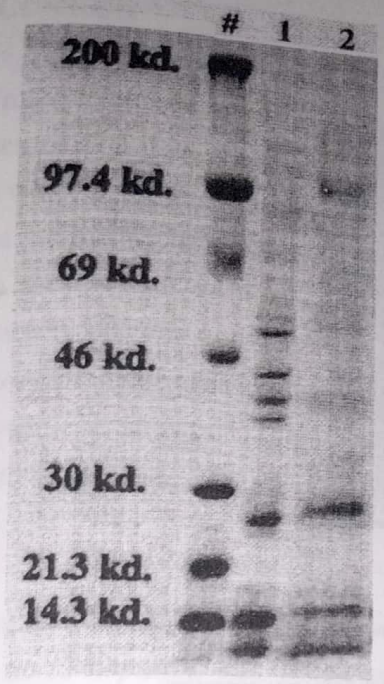

Fig. (1): SDS-PAGE of separated whole worm antigens of :- 1- *F. hepatica* 2- *F. gigantica.* #- Amersham marker 200-14.3 kd.

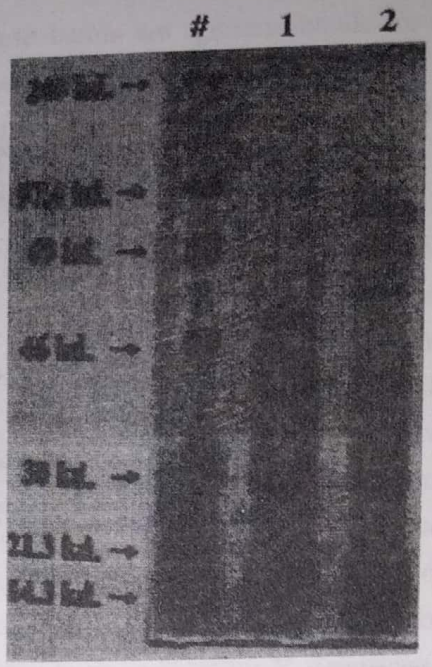

Fig. (2): SDS-PAGE of separated excretory-secretory antigens of :- 1- *F. hepatica* 2- *F. gigantica.* #- Amersham marker 200-14.3 kd.

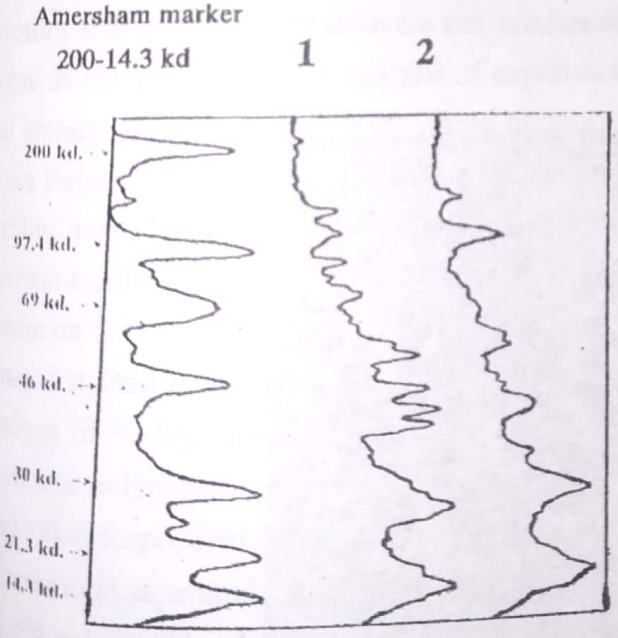

Fig. (3): Densitometry analysis of separated whole worm antigens of:- 1- *F. he-patica* 2- *F. gigantica.*

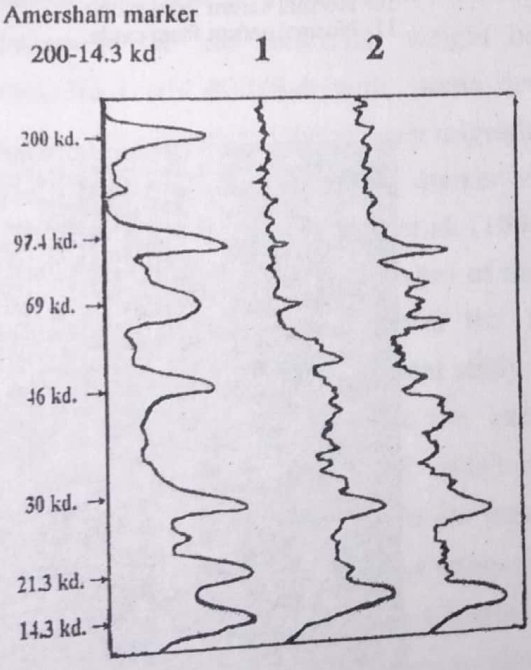

Fig. (4): Densitometry analysis of separated excretory-secretory antigens of:- 1- *F. hepatica* 2- *F. gigantica.*

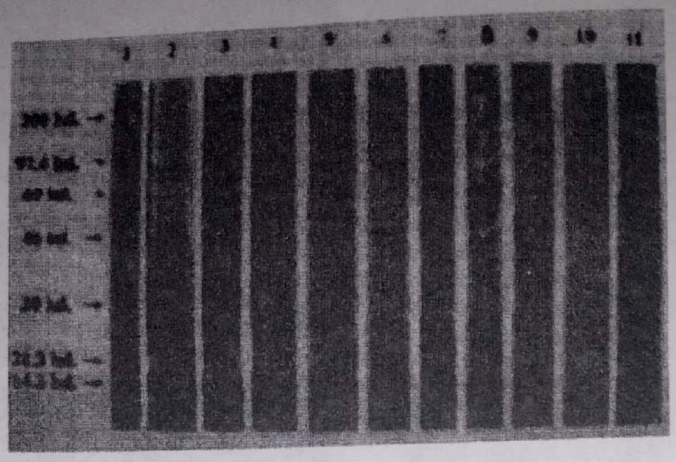

Fig. (5): Enzyme-Linked Immuno Electrotransfer Blot analysis between excretory-secretory antigens of *F. hepatica* and *F. gigantica* and sera of :-
1- Amersham rainbow marker ranged from 14.3-200 kd.
2- Serum of experimentally immunized rabbit with whole *F. hepatica* antigen
3- Serum of experimentally immunized rabbit with whole *F. gigantica* antigen
4&5- Serum of cattle infected with *F. gigantica* based on post mortem examination
6&7- Serum of cattle infected with *F. hepatica* based on post mortem examination.
8&9- Serum of cattle infected with *F. hepatica* based on post mortem examination and seemed to be mixed infection.
10- Normal serum from rabbit.
11- Normal serum from cattle.

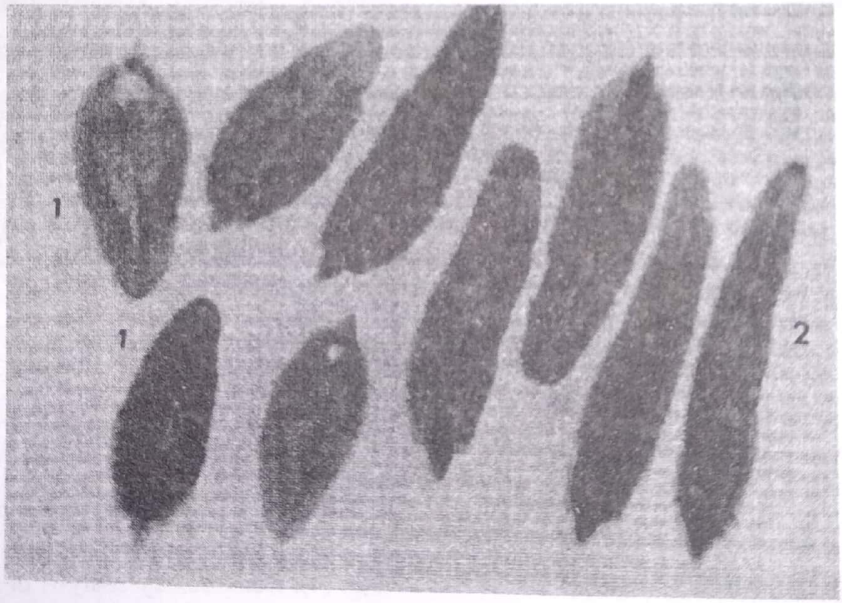

Fig. (6). Collected worms of :- 1- *F. hepatica* 2- *F. gigantica*

DISCUSSION

The main protein concentration of whole worm antigen of *F. hepatica* was determined as 85 mg/gram while it showed 42 mg/gm in case of *F. gigantica* indicating the reverse relation-ship between the size of worm and its protein content .The main protein concentration of excre-tory-secretory preparation of *F. hepatica* was 60 mg / gram while it showed 52mg /gm in case of *F. gigantica* indicating the nearly the same level on host.

Several studies have been performed on the incidence of *F. hepatica* and *F. gigantica* in Egypt and other countries based on the coprological examination, post mortem examination and the distribution of liver flukes in endemic areas without differentiation between Fasciola species. This paper focused on the whole protein analysis, excretory-secretory antigen analysis and immunodiagnosis using western blot analysis of experimental immunized rabbit sera with whole protein of each Fasciola species as well as the sera from naturally infected cattle with fascioliasis. The whole protein banding patterns of both species were different on 5-15 gradient SDS-PAGE gel. The commassei stained banding patterns for whole worm antigen of *F. hepatica* and *F. gigantica* showed common polypeptide approximately at 27.75kd. and 28kd. respectively, representing nearly 10 % of their total separated sample. This was followed by the polypeptide at 14.1kd. and 14.4kd. representing nearly 7% and 10% respectively of total separated protein sample and this suggested that these bands are generic bands for both Fasciola species profile. On the other hand, the study declared that the polypeptide bands at 33.9kd, 41.4kd., 47.2kd. were specific for *F. hepatica* while the polypeptide bands at 34.5kd., 48.5kd. and 105kd. were appeared specific for *F. gigantica*. The analysis of excretory-secretory antigen revealed that the predominant common bands for both parasitic species were also clustered at 14kd. and 28kd. where the first band was clustered up to 21kd. and the second was clustered up to 40kd. in both species. Moreover, the separated *F. hepatica* excretory-secretory antigen was clustered approximately at 47kd. up to 49kd. so, it was suggested to be specific for *F. hepatica* while the polypeptide bands at 56kd., 68kd. and 87kd. seemed to be specific for *F. gigantica*. The pres-ent analysis agreed with finding of Qureshi et al. (1995) who identified the excretory-secretory protein banding battern for *F. hepatica* on SDS-PAGE as clusters in the low molecular weight between 10kd.-30kd. and 40-60kd. with some deviation because they compared the antigen migration distance to known molecular mass marker visually and not by densitometry. Cervi, et al. (1996) prepared the excretory-secretory antigen of the same species at 10,000xg and observed the 14.4kd, 47.2kd. and 33.9kd as in the present study for the whole *F. hepatica* antigen but the excretory-secretory product in our work didn't include 33.9kd. This may be attributed to the present ultracentrifuging of the excretory-secretory preparation at 60, or may be due to the locality species difference. The excretory-secretory antigen of *F. gigantica* antigen showed the polypeptide bands

at 14.6kd., 27.8kd. of whole antigen and clusters of bands at 56.3kd., 68kd., 87.2kd. which didn't noticed in case of *F. hepatica* and this can be used as species fingerprint differentiation. Our finding agreed with the integration of Mbuh and Fagbemi (1996), in which, the immunoblot of infected sera recognized three polypeptides in the rang of 42-80 kd. as early as 2 weeks after infection. In our work the western blot analysis cleared the response to excretory-secretory antigens of *F. hepatica* and *F. gigantica* with the sera from experimentally immunized rabbit as well as naturally infected cattle. It showed that, the two bands at 14.5kd. and 28 kd regions were specific for both Fasciola spp. One band 47 kd. was specific for the *F. hepatica* which was probably similar to the 50kd. and 46kd. (Dumenigo et al. 1996 and Qureshi et al. 1995). The western blot analysis showed five prominent bands represented immuno-reaction between the separated excretory-secretory antigens of *F. gigantica* and experimentally immunized rabbit sera with whole F. gigantica antigen. Three bands at 57kd., 69kd. and 88kd. were observed specific for F. gigantica, whereas the common bands at 14.5kd., 28kd were detected at the same molecular weight as in F. hepatica. This suggests that the latter two bands are generic immunoreactive bands for Fascioliasis. This finding agreed with Fagbemi and Guobadia (1995) who used 28kd. cysteine protease for immunodiagnosis of *F. gigantica* adult worms in cattle, sheep and goat. Guobadia and Fagbemi (1997) showed that, the polypeptide 69kd. was detectable from the fourth week of sheep infection of *F. gigantica* and showed the immunoreactive bands of sera obtained from infected cattles with the corresponding species compared with controlled immunized rabbit sera indicating the common immunoreaction also at 14.5kd. and 28kd. Only two sera of clinically infected cattle with *F. hepatica* based on postmortem examination recorded the generic bands 14.5kd., 28kd. in addition to the polypeptide band at 47kd. which was also specific to *F. hepatica* and the band at 69 kd. which related to *F. gigantica*. This ob-servation suggests as, the presence of mixed infection with both types of Fasciola. From previously illustrated data, it was also noticed that, the separated crud parasitic major protein bands may differ than the major immunoblot one. This notice suggests as , not all separated crud para-sitic protein activate the immune system of the host body and this agree with Frederick, et al., (1994). Moreover all polypeptide bands of infected probed cattle sera appeared more faint than in case of rabbit sera and this may be due to the chronic stage of infection. The rate of in-fection in this study was 6% and this don't consider as a fit incidence because the blood samples was collected from different and unknown localities to the abattoir. This immuno diagnostic method offered an alternative as a supplement to the conventional coprological and serological diagnosis of fascioliasis when there is a need to differentiate fascioliasis in early stages of the disease.

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