DIAGNOSIS OF FASCIOLA INFECTION BY DOT ELISA TECHNIQUE USING SDS-PAGE ELUTED EXCRETORY SECRETARY (ES) PROTEIN FRACTIONS.

By

Nasr M. El-Bahy

Dept. of Parasitology, Fac. Vet. Med. Minufiya University

SUMMARY

Three F.gigantica ES protein bands of molecular weight (MW) ranged from 14-20 KD, 25-30 KD and 45-65 KD, were eluted after fractionation of the parasite antigen using SDS-PAGE. The eluted KD protein bands were concentrated and evaluated in diagnosis of Fasciola and their cross reaction with other parasitic infection in animals of known parasite eggs/gram feces using Dot ELISA technique.

Protein bands in the range of 14-20 KD and that of 25-30 KD were markedly specific and sensitive in diagnosis of different level of anti-Fasciola antibodies (Ab) in sera of infected animals. These two groups of bands were able to exclude cross-reaction between anti-Fasciola Ab and other parasite recorded in feces of different groups of animals include Paramphistoma spp. Monezia spp, PGI, and Toxocara vitullorum either in single infection or in mixed condition with Fasciola eggs. While that of 45-65KD appear less specific than the other previous mentioned bands

Protein bands in the range of 25-30 KD appear more sensitive than the other protein bands in detection of ant-Fasciola Ab at higher serum dilution. Dot ELISA technique was proved to be more economic and easy in application. The dotted very small amount of antigens can be stored in freezer and used at request in diagnosis of large numbers of samples.

INTRODUCTION

Several previous studies were done to induce early accurate diagnosis of *Fasciola* infection with special interest to exclusion of the cross reacted parasites using different serological techniques as IHA and ELISA. In these tests different *Fasciola* antigens (Ag) were used enclude crude worm Ag (Mansour et.al,1983), excretory secretary (ES) Ag (Santiago & Hillyer, 1986, Mousa, 1992, Shalaby, 1998 & 2002), egg antigens (Hassan et.al.,1989). Each author clarify some advantages and dis-advantages for his technique.

It was commonly understand that ELISA test is a sensitive and simple method for semi-quantitative determination of antibodies as mentioned by (Kaddah et al., 1992). Their specificity in exclusion of cross reacted parasites was depends mainly on the degree of specificity and purity of the used Ag, and history of the tested sera (El-bahy, et.al.1999).

Pappas et al (1983) subsequently described as more rapid, economic, direct and visual read, improved ELISA technique for the diagnosis of visceral leishmaniasis as microenzyme-linked immunosorbent assay (dot-ELISA). Zimmerman et al (1985) and De Morilla et al (1989) used this technique for diagnosis of Ovine fascioliasis using crude antigen. They cleared that nanogram quantities of parasite antigen were dotted onto very small piece of nitrocellulose membranes considered enough to obtain marked accurate diagnosis for the parasite directly.

Gorman et.al., (1994) fractionate *F.hepatica* ES Ag using Sodium dodochyl-sulfate-Poly acrylamide gel electrophoresis (SDS-PAGE) and select protein bands of 16, 26-28, 35-36 and 56-58 KD as specific bands for diagnosis of *Fasciola* infection in sheep with different degrees of specificity. In the same time, Intapan et al.,(1998) determine that the most specific protein band in *F.hepatica* ES Ag. is that of 14-38 KD protein bands and the most specific one is that of 27 KD .

In the present study, Sodium dodochyl-sulfate-Poly acrylamide gel electrophoresis (SDS-PAGE) were used in separation of special purified fractions of *F.gigantica* ES Ag. The most common previously mentioned *Fasciola* protein bands were cut out from the gel. The different antigenic bands in the given piece of gel were eluted and concentrated. Their value in diagnosis of Fasciola infection and exclusion of cross reaction with other related parasites were evaluated using Dot ELISA technique versus animal sera of known parasite fecal infection history as will as rabbit hyper immune and negative sera as control.

MATERIALS AND METHODS

1- The selected animals and control samples:

The samples used in the present study were collected from living natural infected buffaloes in private farm at Fayoum Governorate . These animals were put under continuous observation for two successive years where their feces were examined monthly for evaluation of their parasitic infection during other related work plan. Other than *Fasciola*, different parasitic egg count did not recorded due to their marked fluctuation during the period of animal observation.

A total of 86 selected serum sample of known parasite infection history were tested. From each animals rectal fecal and serum sample was collected regularly for chick on the condition of infection..

The collected sera were arranged in 10 groups, according to the type of natural infection which detected in animal by fecal examination. The first group (G-1) is 10

serum samples of buffaloes harboring *Fasciola* eggs only in their feaces (5 of low EPG[1-4 egg] & 5 of high EPG[5 eggs or more] according to Malone et. al. ,1987). G-2 is 8 buffaloes harboring *paramphistomum* spp. eggs only. G-3 is 6 buffaloes infected with *Moniezia* species eggs only. The fourth group (G-4) is 10 buffaloes calves infected with *Toxocara vitulorum* eggs only. G-5 is 10 serum samples collected from buffaloes harboring eggs of different gastro-intestinal parasite *(G.I.P)*. G-6 is 5 serum samples of animals shed *Fasciola* and *Paramphistomes* egg together. G-7 is 7 serum samples of animals shed both of *Fasciola* and *Moniezia* eggs in their feaces. G-8 is 10 samples of animals shed both of *Fasciola* and P.G.I. eggs in their feaces. G-9 is 10 serum samples of buffaloes shed eggs of *Fasciola, Paramphistomum* and *G.I.P.* simultaneously in their feces. G-10 is 10 serum samples collected from young (6-7weeks old) buffalo calves as non-infected control for the test (these animals still free from any parasitic infection for more than 3 months after this). Also, two hyper immune Rabbit sera were prepared and used as reference control positive sera.

Antigen preparation:

***F.gigantica* excretory secretory antigen.(E.S.Ag):**

F. gigantica ES Ag was prepared from living flukes collected from fresh condemn buffaloes livers according to River Marrero et al., (1988). The clean active worms were incubated (40 worms per 100 ml) for 3 hours at 37° C in PBS (7.4 pH). The supernatant was separated after centrifugation at 5000 rpm at 4 °C for 1 h. Their protein content was increased by dehydration using polyethyleneglycol in molecular porous membrane tubing 6-8 MW cut off (Spectrum Medical Inc., Los Angeles, CA 900060). After determination their protein content by method of Lowry et al. (1951), The antigen was allocated into 1ml vial and stored at –20 °C until use.

Reference control hyper-immune rabbit serum:

Rabbit hyper-immune sera as reference control were raised against *F. gigantica* ES Ag., according to Langley and Hillyer (1989). Two, 2-month-old parasite free white New Zealand rabbits were used. The initial subcutaneous injection was in Freund's complete adjuvant. About 1.2 mg of protein was mixed 1:1 with Freund's complete adjuvant and injected one time subcutaneously at different places in the back of the rabbit. After 3 weeks another 1.2 mg of protein was mixed 1:1 with Freund's incomplete adjuvant and divided into 3 doses, injected intramuscularly at biweekly intervals. Rabbits were bled from the ear vein for serum collection 10-14 days after the last injection. The collected sera were stored at –20°C until used.

Production and separation of specific selected *F.gigantica* protein bands:

A- Fractionation of *F.gigantica* ES Ag. using (SDS-PAGE)

SDS- PAGE was performed according to Laemmli (1970) in 10 % polyacrylamide gel slabs in Tris-glycine buffer, pH 8.3. *F.gigantica* ES Ag. were diluted 1:1 in sample buffer consisting of 31.25 mM Tris base 1 % SDS, 2 % 2-mercaptoethanol and 5 % glycerol. The stacking gel consisted of 5 % acrylamide prepared in 12.5 mM Tris-HCL buffer (pH 6.7) containing 1 % SDS. All reagents

were commercially supplied (Sigma chemical Co.). Low and high MW standard were employed (Sigma SDS-100B). Gel strip containing the standard and part of *F.gigantica* fractionated Ag were cut out fixed and stained with Commasi-blue stain according to the method of Tsai and Frasch (1982) These strips were retained again to its original position for determination of the sit of the selected protein bands.

B-Isolation of selected protein fraction from SDS-PAGE by electro elution:

Continuous 10 % gels (1.5 mm thickness) were isolated with 1 mg ml^{-1} of *F.gigantica* ES proteins. Individual slots in the same gel were used to electrophoreses the molecular weight (MW) standards. Once the gel ran its full length, strips with the MW standards were cut and rapidly stained with Coomassie blue to determine the region where the antigens of interest would be according to the associated approximate MW.

As in fig (1), three zones in the gel to contain the protein of 45-65 KD(a), 25-30 KD(b) and 14-20 KD(c), were cut out horizontally across the whole gel. Each gel strip were transferred separately to elution tube membrane 6-8 MW cut off (Spectrum Medical Inc., Los Angeles, CA 900060).The tube were filled with PBS (pH 7.4) and kept in Bio-Rad elution unit at 10V, 100mA over night at 4 °C.
The gel material were removed and the volume was reduced using polyethyleneglycol in molecular porous membrane tubing 6-8 MW cut off according to Katrak et al. (1992). The protein contents of the eluted concentrated materials was determined and kept in 1 ml vial at –70°C till use.

Other antigens used in the test:-

In order to evaluate the presence of cross reacted anti-bodies in each of the tested serum samples, four types of other common parasites crud antigens were prepared include *Paramphistomum spp* adult worm, *Moniezia scoleses, Toxocara vitulorum* anterior end and *Homonchus* species. The worms were collected from naturally infected animals slaughtered in Cairo abattoir .

According to Hillyer & Santiago (1977) , the fresh active worms were washed out repeatedly in PBS (pH7.4) for 1hour. The worms or the selected part of the worm were homogenized (ULTRA-TURRAX Janke & Kunkel KG) at 6000 rpm for 20 minutes in ice bath. The homogenized samples were then subjected to high-speed centrifugation (20 000 rpm) for one hour at 4 °C. The supernatant was separated as crude soluble antigen. The protein content of each antigen was determined before their storing in small vial at –70 °C using method of Lowry et al. (1951).

Dot ELISA technique:

The technique described by Shaheen et.al, (1989) after modification by De-Morilla et al, (1989) was adopted. One microlitre (1ul) of the tested antigens (1.0mg protein/ml) in PBS-T(pH 7.4) was dotted on 6-mm diameter nitrocellulose membranes discs. A checked mark was made to identify the surface for antigen application.The discs were dried at 56 °C for 10-15 min and placed into flat bottom of polystyrene 96 well ELISA plate. Non-specific binding sites were blocked by adding to each well 100 ul 3% BSA-PBS-T for 30 min. The discs were washed after this in PBS-T and dried again. Before use, the discs was wetted in PBS-T and air

dried. Subsequently, 20 ul of diluted sera (in PBS-T) was applied on top of the dotted antigens in the well in doublects. The plates were incubated at room temperature for 1h..The discs were washed again 3-5 times in PBS-T. 50ul of horseradish peroxidase conjugated goat anti-bovine IgG (Sigma) and goat anti-rabbit IgG (Sigma) diluted at 1:1000 , was added to each well and incubate for 30 min. at room temperature. After 3-5 time washing, the discs were immersed each in 50 ul of freshly prepared substrate solution (4-chloro 1-naphthol 340ug/ml substrate buffer with 0.03% hydrogen peroxide solution). Color developed within 15 min and the discs were then washed with water and air-dried. The intensity of the blue-purple colored was judged by the naked eye and evaluated as in plate 1-b

For selection of the optimum condition for running the test, three antigen concentrations were test as 50ng, 100 ng and 200 ng per dot. Serum was tested at 1:50, 1:100 and 1:250 dilution in PBS-T, While the conjugate was applied for each case at 1:500, 1:1000 and 1:2000 for 30 minutes at room temperature.

Tested serum was used first at 1:100 dilution then serial dilution as 1:100, 1:250, 1:500 and 1:1000 were used for evaluation the sensitivity of the most immuno-dominant eluted protein fraction after this.

RESULTS

Optimization of dot-ELISA conditions:

An optimum concentration of 100-120 ng of tested antigen per dot were enough to detect specific antibodies of the target antigen, at 1:100 serum dilution for 1 hour and 1:1000 conjugate for 30 minutes at room temperature. These were found optimum for marked differentiation between positive and negative serum samples by naked eye.

In the present work, specificity and sensitivity of three selected groups of *F. gigantica* ES eluted protein bands at molecular weight (MW) ranged from 45-65 KD, 25-30KD & 14-20 KD (fig 1) were tested in diagnosis of *Fasciola* infection in buffaloes sera (at1:100 dilution). This aimed to define the most specific and sensitive eluted KD protein band (s) that able to induce accurate diagnosis of fascioliasis and in the same time exclude any cross-reaction with other parasites at the level of Ag-Ab reaction using Dot ELISA technique (fig 2).

The results displayed in table (1) cleared that 2 of the three selected protein band (s) group (25-30KD & 14-20 KD) were cleared marked specificity (100%) toward detection of anti *Fasciola* anti bodies (Ab) in all tested sera in group (1) or in mixed infection with other parasites as in G-6 (with *Paramphistomum)*, G-7 which infected with *Moniezia*, G-8 infected with *G.I.P.* or even with more than one parasites as in G-9.

In the same times these two KD MW group of bands did not induce cross reaction (100% specificity) with other parasitic Ab which present in sera of animal as *Paramphistomum* (G-2), *Monizia* (G-3), *T.vitulorum* (G-4) and *G.I.P.* (G-5).

The third selected *F.gigantic* ES protein bands group of MW45-65 KD, appear of low specificity than the other two groups of protein bands. This groups of bands (45-65KD) were induce high specificity (100%) for detection of anti *Fasciola* Ab in sera of buffaloes infected with *Fasciola* only (G-1) or even with other parasites (G-6, G-7, G-8 & G-9).

On the other hand these groups of bands (45-65KD) were cross reacted by different degrees with antibodies of other parasites even with absence of *Fasciola* infection in these sera. The specificity of these bands reached 75% in presence of anti-*Paramphistomum* Ab (G-2), 83.33% in presence of anti-*Monezia* Ab (G-3) and 90% in case of infection by *T.vitulorum* or *P.G.I.* (G-4&G-5) , table (1).

In comparison between these purified fractionated eluted *F.gigantic* ES bands antigen and the other tested crude antigens, 40%, 30%, 20% and 20% from *Fasciola* infected buffaloes (G-1) were cross reacted with *Paramphistomum , Monezia, T.vitulorum and Haemonchus spp.* crude antigen respectively.Also, 37.5%, 25% and 25% from *Paramphistomum* infected buffalo (G-2) were cross reacted with *Monezia, T.vitulorum, Haemonchus* spp. crude antigen respectively.

For the same point of view, 33.3% from sera of animal shed *Monezia* spp. eggs only in their feaces (G-3) were cross reacted with *Paramphistomum , T.vitulorum and Haemonchus* spp. crude antigen .Also 30%, 20% and only 10% from sera of calves infected by *T.vitulorum* worms only (G-4) were cross reacted with *Paramphistomum, Monezia and Haemonchus* spp. crude antigen respectively. While 30%, 40%, 20% and 20% from sera of buffaloes harboring *P.G.I.* eggs only in their feaces (G-5) were cross reacted with *Paramphistomum , Monezia, T.vitulorum and Haemonchus* spp. crude antigen respectively.

In the same times tested crude Ag. of *Paramphistomum , Monezia, T.vitulorum and Haemonchus spp.* were failed to induce absolute specificity in diagnosis of their target anti-bodies in sera of buffaloes infected with other parasites and beside *Fasciola* as in groups 6, 7, 8 and 9 in the same table.

On the other hand, the control reference anti-*F.gigantica* ES hyper-immune Rabbit serum (2 samples) showing absolute specificity in detection of the 3 fractionated, eluted *F.gigantica* ES protein bands (45-65 KD, 25-30KD and that of 14-20KD) with no cross reaction with other parasite antigens. Also non-of all tested antigen produce any type of reaction when tested versus the control negative buffalo calves sera as in table (1).

For this reason data in table (2) clarify the sensitivity of these two protein bands group (14-20 KD, and 25-30KD) in detection of low level of anti- *Fasciola* Ab by serial dilution of known *Fasciola* infected buffaloes and hyper immune Rabbit sera using Dot-ELISA technique.

The results cleared that both KD MWprotein band groups (14-20 KD, and 25-30KD) were succeed in detection of anti-*Fasciola* Ab in different tested sera till 1:250 dilution. With increasing the serum dilution to 1:500, the selected protein bands of MW 25-30 KD appear more sensitive (100%) than the other(14-20) MW groups (60-80% sensitivity) especially in sera of animals shed other parasite eggs with *Fasciola* eggs. The same phenomenon still true under the condition of this Dot ELISA test with increasing the serum dilutions to 1:1000. The eluted Ag of 25-30KD,MW still proved high sensitivity (100%) in buffaloes infected with low level *Fasciola* eggs (1-4 E.P.G) but by lower sensitivity (80%) in case of buffaloes sera infected with *Fasciola* eggs 5 E.P.G. or more. In the same time the sensitivity of this band (25-30 KD) was also decreased to 60-80% at this serum dilution (1:1000) in detection of anti-*Fasciola* Ab in buffaloes harboring other parasite eggs beside *Fasciola* eggs (table 2).

The bands of MW 14-20 KD showing lower sensitivity (40-80%)for diagnosis of Anti-*Fasciola* Ab at serum dilution (1:1000) in different tested animal sera as in table (2).

On screening the sensitivity of these 2 MW eluted protein bands (14-20 KD, and 25-30KD) versus rabbit hyper immune sera, both groups still react sensitive with two tested rabbit sera till dilution 1:500. At serum dilution 1:1000 the protein bands in the range of 25-30KD appear more sensitive (100%) than the other one (14-20KD) which give lower sensitivity (50%) as in table (2).

Protein bands group of MW 14-20 KD and 25-30KD appear more sensitive and specific in determination of anti-*Fasciola* anti-bodies. The antigen of 25-30KD has absolute sensitivity (100%) than the other one till 1:500 serum dilution. The protein MW bands of 25-30 KD appear more sensitive in detection of low level of anti-Fasciola Ab than that of 14-20 KD. This sensitivity still even with increasing the serum dilution till 1:1000 in animals of low *Fasciola* eggs/gram and in tested hyper-immune rabbit sera. This level of sensitivity was decrease to 60-80% in animals of high *Fasciola* EPG and in animals infected by other parasites with Fasciola.

DISCUSSION

Cross-reaction between *Fasciola* species and other parasites still as a questionable point making some difficulties in the accurate evaluation of the infections status of suspect animals using some serological techniques, specially under the level of field collected polyclonal sera. Where accuracy of these tests was affected markedly by degree of purity and specificity of the used antigens. More accurate results can be obtained using Enzyme Linked Immune Transfer Blot (EITB) but this technique usually non-practical for current field application in comparison with ELISA technique (Ibarra et. al., 1998)

According to De Morilla et. al, (1989), ELISA is sensitive serological test able to analyzing many samples simultaneously but it need some sophisticated equipment. The new modified Dot-ELISA was more economic , more suitable for

accurate diagnosis using very few amounts of reagent specially in case of small amount of valuable purified antigen, and very convenient for field study where the results can be read visually.

The present study evaluated the efficacy of Dot ELISA using 3 selected groups of *F.gigantica* SDS-PAGE eluted fractionated ES antigen in three MW ranges (45-65 KD, 25-30 KD and 14-20 KD) in diagnosis of *Fasciola* infection after concentration of the eluted fractions using Dot ELISA technique. Selection of these antigens was depends on previous publish work on *F.hepatica* (Gorman et.al, 1994 & Intapan et.al, 1998) and *F.gigantica* (Moussa,1992 & Intapan et al, 1998).
In the same time, crude antigens for the parasites that present in feaces of selected animals (as *Paramphistomum, Moniezia, T.vitullorum* and *Haemonchus* spp) were used simultaneously as reference for detection of the cross reacted anti-bodies in tested sera.

Specificity of these 3 selected MW protein bands in detection of ant-Fasciola Ab in natural infected buffaloes (either singly or in association with other parasites) was tested using modified Dot ELISA technique. Two of the three selected protein bands (14-20KD & 25-30KD) succeed in induction of absolute specificity (100%) in diagnosis of anti-*Fasciola* Ab in all of the tested serum samples at 1:100 dilution. The third protein bands of 45-65KD MW showing lower specificity reached to 83.3%-90% where it falsely react with one animal from groups infected by *Monezia, T.vitullorum* & *G.I.P.* and didn't harbor *Fasciola* eggs in their feces. This condition did not considered as inapparent infection in these animals where this mild level of cross reaction did not recorded with the other two purified *Fasciola* antigens in the same animals The marked specificity of these two purified *Fasciola* bands antigens are appear in the same MW range described previously by Gorman et al. (1994) and by Intapan et al. (1998) using EITB technique.

The purified eluted *F.gigantica* protein bands under the condetion of the used technique succeed in exclusion of cross reaction that previously mentioned with crude antigens between *Fasciola* and *Paramphistomum* by Guobadia and Fagbemi (1996) and also that between *Fasciola, Monezia* and *Haemonchus* that described previously by Aly (1993). This elimination of cross reaction between *Fasciola* and these parasites may be due to degree of purity and specificity of the selected MW protein bands. This came in agreement with (El-Bahy et al, 1999).

The test cleared failure of some crude Ag used (of other parasites) in induction of absolute specificity toward their target anti-bodies in tested sera. This may be due to low Ab titer in sera of these animals as a result of chronic infection in these cases (Ford et al , 1987 and Khalil et al, 1990).

The second part of the present study was focused on more characterization to the diagnostic value of the two specific *F.gigantica* bands group (25-30 KD & 14-20 KD MW) from the aspect of sensitivity in diagnosis of *Fasciola* infection at different serum dilutions.

The marked sensitivity of 25-30 KD antigens than the other one was more clear in using this KD in detection of the anti- *Fasciola* Ab in hyper-immune rabbit sera where the 14-20 KD fail to detect these Ab at 1:1000 serum dilution in one of the two tested serum samples. Failure of the selected Ag in detection of Fasciola infection in some animals may be related to presence of low anti-bodies titers which usually associated with chronic infection. Superiority of Fasciola Ag fractions in the range of 25-30 KD in accurate diagnosis was proved previously by several authors as Santiago & Hillyer (1986) for (23-28 KD) and Intapan et al, (1998) for 27 KD using EITB technique.

In the same time some author clarifying the role of other KD in diagnosis of fascioliasis as 17 KD (Hillyer & Galanes, 1988), 16 KD (Gorman et al., 1994) and 14.4 KD (Intapan et al., 1998). All of these fractions were in the range of the second selected group of bands (14-20 KD) of the present study. These authors used EITB technique and agreed with superiority of the high MW group of bands than that of low MW rang.

For conclusion, using Dot ELISA technique cleared that both selected purified *F.gigantica* ES fractionated eluted protein band (s) groups of MW range of 25-30KD & 14-20 KD, were succeeded in accurate diagnosis of fascioliasis and exclusion of cross reacted antibodies of other parasites. The protein band (s) groups of MW range of 25-30KD appear more sensitive than the other one.

The new approach adopted in the present paper felicitate commercial use of small amount of purified specific antigen in induction of accurate diagnostic test (Dot ELISA) more easly than EITB and can be read visually.

REFERENCES

Aly, M.E.M. (1993): Some biochemical and serological studies on gastrointestinal; helminth infections in cattle and buffaloes in Dakahlia Governorate. Ph. D. Thesis, Fac. Vet. Med., Cairo Univ.

De Morilla, A.C., Gomez, A. Bautista, C.R. and Morilla, A. (1983): Evaluacion de un antigeno somatico y uno metabolico de Fasciola hepatica en diferentes pruebas immunologicas par el diagnostico de la fascioliasis en bovinose. Tec. Pecu. Mex., 44: 41-45

El-Bahy, M.M.; Hassan, A.A.; Shalaby, S.I. and Shalaby, H.A. (1999): Relation between Fasciola gigantica serum antibody titer and coprological parasitic infection in cattle and buffaloes using ELISA and IHA tests. Beni-Suef Vet.Med.J., 9 (3-A): 201-214.

Ford, M.J., Taylor, M.E. and Bickle, Q.D. (1987): Re-evaluation of the potential of F. hepatica antigens for immunization against *S. mansoni* infection. Parasitol., 94: 27-336.

Gorman, T.; Concha, V.; Fredes, F.; Ferrera, A.; Valdes, A. and Alcaino, H. (1994): Detection of antigens with diagnostic potential in animals with *Fasciola hepatica* infections. Parasitologia al Dia, 18: 26-32.

Guobadia, E.E. and Fagbemi, B.O. (1996): Detection of circulating fasciola gigantica antigen in experimental and natural infections of sheep with fascioliasis. Vet. Parasitol., 65:29-39

Hassan, M.M., Farghaly, A.M., El-Gamal, R.L.R. and El-Ridi, A.M.S. (1989): Cross-reaction in immunodiagnosis with *schistosoma, Fasciola* and *Heterophyes* using ELISA. J. Egypt. Soc. Parasitol. 19(2): 845-852.

Hillyer, G.V., and Galanes, M.S. (1988): Identification of 17 KDa *Fasciola hepatica* immunodiagnostic antigen by enzyme-linked immuno-electrotransfer blot technique. J.Clinic.Microbiol., 26 (10) : 2048-2053.

Hillyer, G.V. and Santiago de Weil, N.(1977): Partial purification of *Fasciola hepatica* antigen for the immuno-diagnosis of fascioliasis in rats. J.Parasitol., 63 :430-433.

Ibarra, F., Montenegro, N., Vera, Y., Boulard, C., Flores, J. and Ochoa, P. (1998): Comparison of three ELISA tests for seroepidemiology of bovine fascioliasis. Vet. Parasitol., 77: 229-236.

Intapan, P.M.; Maleewong, W.; Wongkham,C.; Tomanakarn, K.; leamviteevanich, K.; Pipitgool, V. and Sukolapong, V. (1998): Excretory-Secretory antgenic components of adult *Fasciola gigantica* recognized by infected human sera. Southeast Asian J.Trop. Med. Public. Hlth., 29 :579-583.

Kaddah, M.H., Maher, K.M., Hassanein, H.J., Farrag, A.T., Shaker,Z.A. and Khalafallah, A.M. (1992): Evaluation of different immunodiagnostic techniques for diagnosis of hydatosis in Egypt. J.Egypt. Soc. Parasitol. 22(3): 653-666.

Katrak, K., Mahon, B.P., Jones, W.C., Brautigam, S. and Mills, K.H. (1992): Preparative separation of foreign antigens for highly efficient presentation of T cells in vitro. J.Immunol. Meth., 156 :247-254.

Khalil, H.M., Abdel-Aal T.M., Makhaled, M.K., Abdallah, H.M., Fahmy, I.A. and El-Zayyat, E.A. (1990): Specificity of crud and purified *Fasciola* antigens in immunodiagnosis of human fascioliasis. J. Egypt. Soc. Parasit,20(1): 87-94.

Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of Bacteriophage T 4 . Nature, 227 :680-685.

Langley, R.J. and Hillyer, G.V. (1989): Detection of circulating parasite antigen in murine fascioliasis by two-ste enzyme-linked immunosorbent assays. Am.J. Trop.Med. Hyg., 41: 472-478

Lowry, O.H.Rosenbrough, N.J.; Farr, A.L. and Randall, R.J. (1951): Protein measurement with Folin-phenol reagent. J. Biol. Chem., 193:265-275.

Malone,J.B., Williams,T.E. and Loyacano,M.S.(1987): Fascioliasis in cattle in Louisiana. Am.J.Vet. Res., 48(7): 1167-1170.

Mansour, N.S., Youssef, F.G., Mikhail, E.M. and Boctor F.N. (1983): Use of partially purified *Fasciola gigantica* worm antigen in the serological diagnosis of human fascioliasis in Egypt. Am. J. Trop. Med. Hyg., 32: 550-554.

Mousa, W.M.A.(1992): Studies on the cross reactivity among some helminths of veterinary and medical importance. Ph. Thesis, Fac.Vet.Med., Cairo University.

Pappas M.G., Hajkowski R., Hockmeyer W.T. (1983): Dot enzyme-linked immunosorbent assay (dot-ELISA): a microtechnique for the rapid diagnosis of visceral leishmaniasis. J. Immunol Methods. 64:205-214.

River Marrero, C.A.; Santiago, N. and Hillyer, G.V.(1988): Evaluation Of immunodiagnostic antigens in the excretory- secretory products of *Fasciola hepatica*. J. Parasitol., 74 (4): 646-652.

Santiago de Weil, N. and Hillyer, G.V. (1986): Isolation of potential serodiagnosis F.hepatica by electroelution from polyacrylamide gels. Am.J.Trop.Med.Hyg.,35:1210-1217.

Shaheen H.I., Kamal, A.K., Farid Z., Mansour, N., Boctor, F. and Woody, N.J. (1989): Dot-Enzyme-linked immunosorbent assay (Dot-ELISA) for the rapid diagnosis of human fascioliasisJ. Parasitol., 75 (4) 549-552.

Shalaby, H.A. (1998): Evaluation of antigenicity and diagnostic value of some F.gigantica antigens Ph.D.. Thesis, Fac. Vet. Med. Cairo Univ.

Shalaby, H.A. (2002): Some epidemiological and serological studies on fascioliasis. M.V.Sc. Thesis, Fac. Vet. Med. Cairo Univ.

Tsai. C.M. and Frasch, C.E. (1982): A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Analytical Biochemistry, 119 :115- 119.

Zimmerman G.L., Kerkvliet N.I. and Cerro J.E. (1985): Blood parameters of lambs experimentally infected with Fasciola hepatica. Int. Goat Sheep Res. (3) 1513-1515.

Table (1): Specificity of *F.gigantica* ESantigen in diagnosis of *Fasciola* infection using Dot ELISA technique at (1:100) serum dilution.

History of tested serum samples		Different antigens used in dot ELISA plate													
		F.gigantica 14-20 KD		*F.gigantica* 25-30 KD		*F.gigantica* 45-65 KD		*Paramphistomes* Crude Ag		*Moniezia* crude Ag.		*Taxacara* crude Ag		Homomchus sp. Crude Ag.	
	No.	No of +Ve	Specifisty %	No of +Ve	Specifisty %	No of +Ve	Specifisty %	No of +Ve	Specifisty %	No of +Ve	Specifisty %	No of +Ve	Specifisty %	No of +Ve	Specifisty %
Buffaloes shed Fasciola. Eggs only(G1)	10	10	100%	10	100%	10	100	4	60%	3	70%	2	80%	2	80%
Buffaloes shed Paramphistomes eggs(G2)	8	0	100%	0	100%	2	75%	8	100	3	62.5%	2	75%	2	75%
Buffaloes shed Moniezia eggs only(G3)	6	0	100%	0	100%	1	83.33%	2	66.6%	6	100%	2	66.6%	2	66.6%
Buffaloes calves shed T.vitullorum eggs only. (G4)	10	0	100%	0	100%	1	90%	3	70%	2	80%	10	100%	9	90%
Buffaloes shed G.I.P. eggs only (G5)	10	0	100%	0	100%	1	90%	3	70%	4	60%	8	80%	8	80%
Buffaloes shed Fasciola & Paramphistomes. eggs(G6)	5	5	100%	5	100%	5	100%	4	80%	1	80%	2	60%	2	60%
Buffaloes shed Fasciola & Moniezia eggs.(G7)	7	7	100%	7	100%	7	100%	2	71.4%	6	85.7%	2	71.4%	2	71.4%
Buffaloes shed Fasciola & G.I.P. eggs(G8)	10	10	100%	10	100%	10	100%	2	80%	3	70%	6	60%	10	100%
Buffaloes shed Fasciola, Paramphistomes & G.I.P.eggs in feaces(G9)	10	10	100%	10	100%	10	100%	9	90%	4	60%	5	50%	10	100%
Control non-infected animals.(G10)	10	0	100%	0	100%	0	100%	0	100%	0	100%	0	100%	0	100%
Anti-Fasciola ES Hyper-immune rabbit sera	2	2	100%	2	100%	2	100%	0	100%	0	100%	0	100%	0	100%

Sensitivity = True positive / (True positive + False negative) Specificity = True negative / (True positive + False negative)
According to Attallah et al (1997).

Table (2): Sensitivity of *F.gigantica* ES antigen in diagnosis of *Fasciola* infection using Dot ELISA technique

Tested serum samples			at 1:100 serum dilution				at 1:250 serum dilution				at 1:500 serum dilution				at 1:1000 serum dilution			
			F.gigantica 25-30 KD		*F.gigantica* 14-20 KD		*F.gigantica* 25-30 KD		*F.gigantica* 14-20 KD		*F.gigantica* 25-30 KD		*F.gigantica* 14-20 KD		*F.gigantica* 25-30 KD		*F.gigantica* 14-20 KD	
Animal status		No.	No.of +Ve	Sensitivity %	No of +Ve	Sensitivity %	No.of +Ve	Sensitivity %	No.of +Ve	Sensitivity %	No.of +Ve	Sensitivity %	No.of +Ve	Sensitivity %	No.of +Ve	Sensitivity %	No.of +Ve	Sensitivity %
buffaloes infected *by Fasciola* eggs only	1-3 EPG*	5	5	100 %	5	100 %	5	100%	5	100%	5	100 %	5	100	5	100	4	80
	Over 4 EPG	5	5		5		5		5		5		5	100	5	80	3	60
buffaloes infected *by Fasciola* and other parasites eggs	*Paramphistomes*	5	5		5		5		5		5		5	100	4	80	3	60
	G.I.P.	5	5		5		5		5		5		3	60	4	80	2	40
	Moniezia sp.	5	5		5		5		5		5		4	80	3	60	2	40
	mixed	5	5		5		5		5		5		4	80	3	60	3	60
Rabbit *hyper immune sera against F.gigantica* ES Ag.		2	2		2		2		2		2		2	100	2	100	1	50
Control non-infected animals		10	0	0	0	0	0		0		0		0	0	0	0	0	0

* EPG= eggs per gram feces.

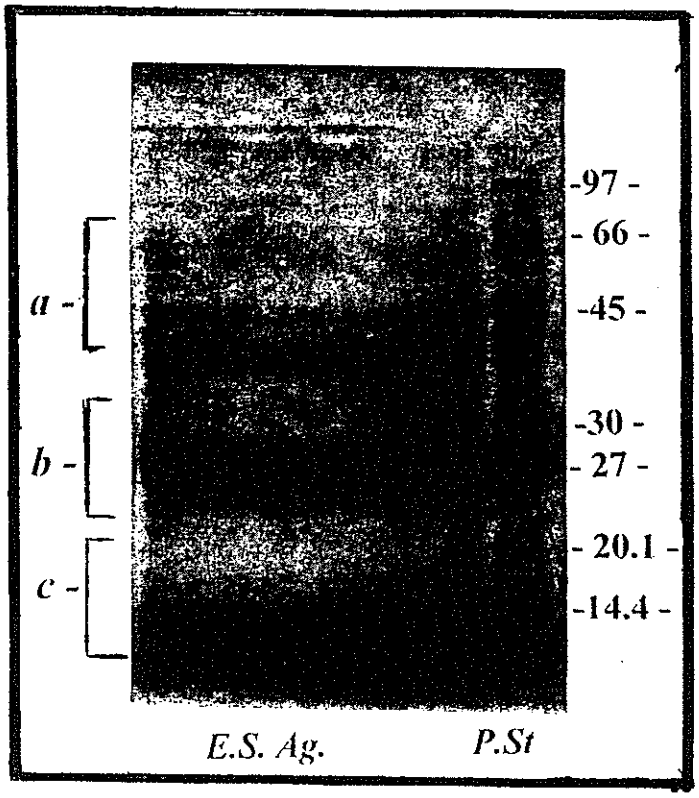

Fig (1) Gel electrophoresis showing the three selected protein bands that used for accurate diagnosis of fascioliasis after elution.
a= 45-65, b= 25-30, c= 14-20 KD, MW
E.S. = Fractionated *F. gigantica* excretory secretory antigen.
P. St = Low M. W. protein standard.

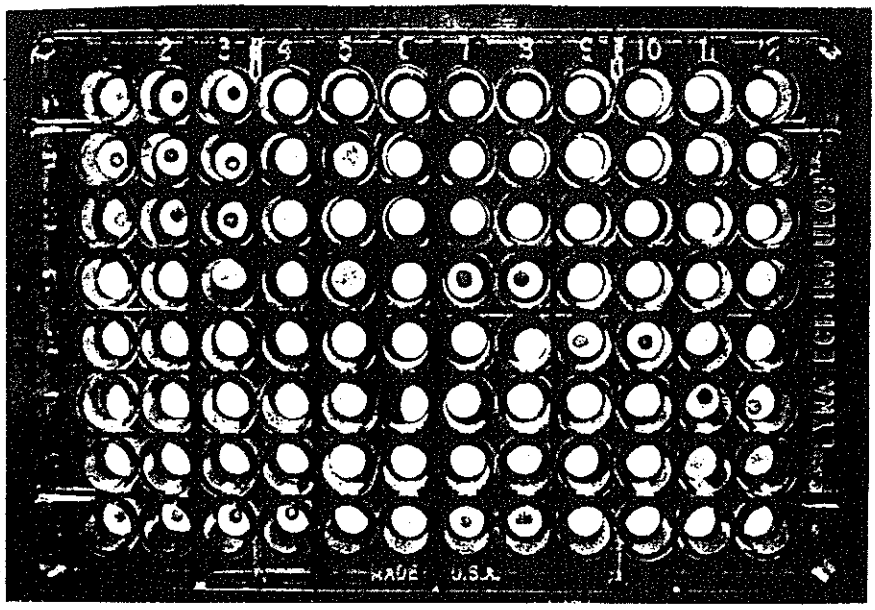

Fig (2) Dot ELISA plate showing markedly defined blue purple circle on area of Ag application which considered as positive, while the diffused pale blue or colorless considered as negative serum sample.

الملخص العربى

تشخيص الاصابة بالديدان الكبدية باستخدام طريقة دوت اليزا والانتيجين المنقى والمعزول بواسطة التحليل الكهربى

نصر معوض الباهى

أمكن استخلاص ثلاثة أنواع من البروتينات النقية عند أوزان جزيئيه مختلفة بواسطة التحليل الكهربي. وقد استخدمت تلك البروتينات النقية كل على حده فى تقييم الإصابة بالديدان الكبدية لحيوانات معلوم مسبقا حالتها من حيث الإصابة بالطفيليات المختلفة. هذا وقد جرى التقييم على أساس التفاعل بين تلك البروتينات كانتيجينات والأجسام المضادة الموجودة فى مصل الحيوانات المصابة بالديدان الكبدية وذلك باستخدام طريقة ال دوت اليزا

وقد اتضح ان البروتين ذى الوزن الجزئي ١٤-٢٠ وأيضا ٢٥-٣٠ كيلودالتون كانا الأفضل والأكثر حساسية فى تشخيص الإصابة بالديدان الكبدية واستبعاد التداخل التفاعلي بين الإصابة بالديدان الكبدية والطفيليات الأخرى التي يمكن ان تتشابه في الاثاره المناعية عند الحيوانات المصابة بتلك الطفيليات. بينما اظهر البروتين ذى الوزن الجزئي ٤٥-٦٥ كيلودالتون حساسية تشخيص اقل من السابقين.

وقد اظهرت الاختبارات بعد تخفيف مصل الحيوانات المصابة بالديدان الكبدية لعدة مرات ان البروتين ذو الوزن الجزئي ٢٥-٣٠ كيلودالتون هو الأفضل على الإطلاق من حيث الدقة والحساسية.

ومن الجدير بالذكر فان طريقة ال دوت اليزا كطريقة للتشخيص تعد من افضل الوسائل التشخيصية فهى تستلزم استخدام قدر ضئيل من مصل الحيوان, وهى حساسة جدا حتى عند التخفيفات العالية للمصل ,وهى وسيلة تطبيقية سهلة حيث يمكن تجهيز واعداد الأقراص المحملة بالانتيجين وحفظها فى الفريزر ثم استعمالها فى اى وقت او استخدامها فى الحقل واخيرا فانها الطريقة التى تستخدم أسس التشخيص المناعية ويمكن قراءة النتائج بالعين المجردة دون الاستعانة بأي أجهزه.