

## **IMMUNOLOGICAL IDENTIFICATION OF *FASCIOLA HEPATICA* ANTIGENS CONTAINING MAJOR HUMAN T-CELL AND B-CELL EPITOPES**

By

**MONA M. MOHAMED, MAGED M. AL-SHERBINY,  
ABLA A. SHARAF AND TAHANI H. ELMAMLOUK**

Department of Zoology, Faculty of Science, Cairo  
University, Cairo, Egypt (email monamoa@hotmail.com)

### **Abstract**

*Fasciola hepatica* whole worm homogenate (Fhwwh) separated fractions were used in enzyme linked immunoelectro-transfer blot (EITB) to identify the antigen(s) which induces antibody formation in human fascioliasis. The immuno-reactive antigens recognized by the infected patients were 25-29 kDa and 12 kDa. Antigens were biochemically purified by model 491-prep cell fraction (BIO-RAD). The capability of the purified target antigens to induce humoral and cellular responses with cells and sera of infected patients was investigated using enzyme linked immunosorbent assay (ELISA) and lymphoproliferative responses techniques. The 25-29 kDa cluster of antigen(s) were found to be more efficient in inducing lymphoproliferative response than 12 KDa, thus, it was considered as the target antigens used in the generation of human monospecific polyclonal immunopurified antibody probes (IPAb). The specificity and immunoreactivity of the IPAb with Fhwwh, *F. hepatica* excretory secretory products (FhESP) and *S. mansoni* adult worm antigen (SAWA) were evaluated by using EITB. Results showed that IPAb was immunoreactive with 25-29 and 12 kDa antigens of both Fhwwh and FhESP. It was concluded that the most immunogenic *F. hepatica* target antigen(s) were 25-29 & 12 KDa antigens and there is a cross-reactivity between 25-29 & 12 KDa antigens in both Fhwwh and FhESP. The

involvement of IPAb in antibody dependent cell mediated cytotoxicity *in-vitro* technique was studied. Results indicated that human neutrophils were more effective in adhering to the IPAb-coated flukes at early stages of development.

## Introduction

Fascioliasis, is a parasitic disease caused by a trematode that belongs to family Fasciolidae known as *F. hepatica* or "liver fluke". The disease is worldwide distributed it is a major cause of morbidity and mortality in domestic ruminants (Hillyer and Apt. 1997). Humans may acquire the infection by ingestion of raw vegetables or water contaminated with metacercariae (Arjona *et al.*, 1995; Saba *et al.*, 2004). Approximately 2.4 million people were infected with either *F. hepatica* or its larger relative *F. gigantica*, mostly within Bolivia, Peru, Egypt, Portugal and China (WHO, 1995). Earlier attempts to isolate the parasite antigens contained in adult *F. hepatica* were first reported by Korach and Benex (1966) whom extracted a lipoprotein from adult flukes. The *F. hepatica* purified antigens have been reported as having potential for antibody detection assays in fascioliasis (Hillyer *et al.*, 1977). *F. hepatica* protein pattern was recently identified by Allam *et al.* (2002). The strength of interaction of antibodies with a *F. hepatica* antigen has been investigated for several years. Studies on humoral immune response manifested during the course of fascioliasis, showed that IgM, IgE, IgG1 & IgG2a antibody isotypes were all elevated in serum of the infected rats compared to serum obtained from control, uninfected rats. Furthermore, an increase in neutrophils and eosinophils were also observed (Pfister *et al.*, 1983; Poitou *et al.*, 1993; Van Milligen *et al.*, 1998). Studies used enzyme-linked immunosorbent assay (ELISA) proved that IgG titers remain elevated in serum throughout the *F. hepatica* infection, even long periods after chemoprophylaxis (Marín, 1992, Haseeb *et al.*, 2003). The previous results implies that *F. hepatica* antigenic products stimulated prolonged immune response (Poitou *et al.*, 1993). Studies showed that *F. hepatica* purified antigens have been reported as having potential for antibody detection assays in fascioliasis. Two antigens, the 12

kDa *Fasciola/Schistosoma* cross-reactive, cross-protective antigen related to the fatty acid binding proteins (FABPs) (Hillyer, 1995) and cluster of antigens (GST & cathepsin proteases) found at the zone 27-30 kD are the most defined antigens showing intense humoral and cellular responses (Piacenza *et al.*, 1999; Hillyer, 1999). The antigenic components of excretory-secretory products of adult *F. hepatica* recognized in human infections was found to be 25 and 27 kDa components. These findings suggested that the 25- and 27-kD antigenic components may be sensitive and specific for the diagnosis of human fascioliasis (Sampaio-Silva *et al.*, 1996). Abdel-Rahman *et al.* (1999) confirm the stability of the 26-28 kDa coproantigen and its usefulness in diagnostic tests for *F. hepatica* infections.

The aim of the study was to identify *F. hepatica* specific antigen(s), that induce antibody formation in human fascioliasis. Then purify biochemically the target antigens and test the potentiality of the isolated antigens in lymphocytes proliferative activity, in order to prepare specific antibody probes that could be used in screening *F. hepatica*  $\lambda$  gt11 cDNA expression library. The immunological efficacy of the prepared antibody probe and its involvement in antibody dependent cell mediated cytotoxicity (ADCC) were also tested.

## Materials and Methods

Thirty-seven individuals (twenty females, seventeen males) were enrolled in this study, and divided into twenty-four *Fasciola hepatica* and six *Schistosoma mansoni* infected subjects inhabiting Abis village, Alexandria G., Egypt, and a control group of seven unexposed, uninfected, healthy subjects. This protocol and the informed consent of patients participating in the study was reviewed and approved by the Ethics Committee in VACSERA (Egyptian Ministry of Health and Population).

Preparation of *F. hepatica* excretory-secretory products (FhESP) and whole worm homogenate (Fhwwh): *F. hepatica* adult worms were collected from the bile ducts of condemned cattle livers at a local abattoir. To remove all traces of blood and bile; the parasites were washed 3-4 times at room temperature

for 1h with 0.01 M phosphate-buffered saline (PBS) [0.12 M NaCl, 0.08 M KCl, 0.08 M  $\text{KH}_2\text{PO}_4$  (Mallinckrodt, Chesterfield, MI, USA) and 0.01 M  $\text{Na}_2\text{HPO}_4$  (SIGMA<sup>®</sup>), pH 7.4]. To prepare the excretory/secretory products (FhESP), worms were then incubated in excess PBS (20 worms/100 ml), supplemented with 2% glutamine (GIBCO BRL, Grand Island, NY, USA) and 2% penicillin/streptomycin (SIGMA<sup>®</sup>), at 37°C in  $\text{CO}_2$  incubator for 3-4h as described by Santiago and Hillyer (1986, 1988) and Zurita *et al.* (1987). The worms were collected and stored in liquid nitrogen until used.

Five grams of worms were thawed, transferred into 5 volumes of 0.01 M PBS [pH 7.4 containing protease inhibitors and 0.1%  $\text{Na}_2\text{N}_3$  (SIGMA<sup>®</sup>)] and homogenized. The homogenate was filtered then sonicated using a Heat System Sonicator Model 375 (Heat system, Plain View, NY, USA). The sonicate was then centrifuged at 20,000x *g* for 15 min. The process of homogenization, sonication and centrifugation was performed at 4°C. The supernatant was assayed for protein content (Bradford, 1976) aliquoted and stored at -80°C until used.

Sodium-Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): Fhwwh and FhESP protein pattern were characterized by (SDS-PAGE) (Tsang *et al.*, 1983) and stained with silver according to Morrissey (1981). The  $M_r$  of the electrophoretic protein bands were determined as described by (Makowski and Ramsby, 1998).

Enzyme linked immunotransfer blot (EITB): This process was carried out as described by Tsang *et al.* (1983; 1985); Hillyer and Soler de Galanes (1988). Briefly after termination of SDS-PAGE, a sheet of nitrocellulose (NC) was placed against the surface of the gel and both were soaked in blot buffer [0.43 M Tris-Hcl pH 9.18, d  $\text{H}_2\text{O}$ , methanol with a ratio of 5:3:2 (v/v/v)]. Electrophoretic transfer of SDS-protein complexes was accomplished in 1h at 1.01 amp (PowerPac 1000 Power Supply, BIO-RAD) and at 4°C. The NC blot was cut into longitudinal 0.2 cm wide strips using a bladeless immunostrip cutter (Novex technology, San Diedo, CA, USA). The NC strips were then incubated with IFS, NHS (each diluted 1:50 in PBS/0.3% Tween-20 and 5% nonfat milk) for 1h with continuous agitation

on a platform shaker (Bellco). Washing was carried out for 3 times (1min each) with PBS/0.3% Tween-20 at room temperature. The NC was then incubated for 1h with conjugate horseradish peroxidase labeled immunoaffinity purified goat anti-human IgG [Kirkegaard and Perry laboratories (KPL), Inc. Gaithersburg, MD, USA] diluted 1:1000 in PBS/0.3% Tween-20. The NC was then washed 3x1 min with washing buffer and 2 times with PBS only. The substrate solution [50 mg of 3,3'-diaminobenzidine (DAB) (SIGMA®), 10 µl of H<sub>2</sub>O<sub>2</sub> (30%) (KPL), in PBS pH 7.2 to 50 ml] was then added. Positive reaction bands usually appear within 10 min and washing with water stopped the reaction.

Isolation and purification of Fhwwh specific antigens: Selected Fhwwh bands were electroeluted using Model 491-prep cell (BioRad) according to manufacturer instructions. Each 491-run was followed by SDS-PAGE and EITB to determine the M<sub>r</sub> of the separated fractions and to show the immunoreactivity of the separated fractions in order to determine the most immunoreactive fraction. Fractions of the same molecular weight were collected from different runs, lyophilized (Freeze dry system LYPH. Lock 4.5, Labcono Co., Kansas city ,MO, USA) assessed for protein concentration and finally stored at -60°C till used. Before usage the lyophilized samples were dissolved in distilled H<sub>2</sub>O and then passed onto PD10 column (Pharmacia) for salt removal.

Lymphoproliferative responses: The Fhwwh and 491-prep cell purified antigens at different concentrations (2.5, 5 and 10 µg/ml) were suspended in 100 µl of sterile RPMI-1640 culture medium (Bio-whittaker, Walkersville, Maryland, USA). The culture media was supplemented with L-glutamine (200 mM), 100 µg/ml penicillin, 100 µg/ml streptomycin and 1 M HEPES buffer (N-2-hydroxyethylpiperazine N'-2-ethane-sulfonic acid) (all from GIBCO BRL). Mononuclear cells (MNCs) were isolated from peripheral blood of patients and controls (Farrant *et al.*, 1980). The viability of MNCs was measured using trypan blue stain. The cells were adjusted at a concentration of  $1.14 \times 10^6$  cells/ml in RPMI-1640 media supplemented with 5% heat inactivated, mycoplasma-and virus-free AB normal human serum (SIGMA). The cells were then cultured in triplicates in a

volume of 175  $\mu$ l/well (200,000 cells/well) into the previously prepared culture plates (Greiner). Negative control consisted of cells cultured alone with complete RPMI-1640 media. The plates were then incubated for 5 days at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> in CO<sub>2</sub> incubator (NuAIRE™ IR Auto Flow, CO<sub>2</sub> water-Jacketed Incubator, USA). On the last day of culture, each well was pulsed for 18h with 0.5  $\mu$ ci of [<sup>3</sup>H] thymidine (DuPont Nensure, Boston, MA, USA). The wells were harvested with a multi-well harvester (Harvester 96, Tomtec, CT, USA) into glass Fiber filters (Filtermat A 102x258 mm, Wallac, Turku, Finland). The dried filters were immersed in scintillation cocktail and the radioactivity was measured in a liquid scintillation counter (Wallac).

Lymphocyte blastogenesis data was expressed as the ratio of mean counts per min (mcpm) of the triplicate experimental (E) cultures to the mcpm of the control (unstimulated) cultures to give an E/C value in cpm. Statistical analysis was carried out between the cells lymphoproliferation of control subjects and the infected patients using Student's *t*-test.

Preparation of human immunopurified antibody (IPAb) probe: *F. hepatica* 491- purified target of antigens (25-29 kDa) were coupled to one gram CNBr-activated Sepharose 4B, according to the manufacturer (Amersham Pharmacia Biotech). Specific antibodies binding was achieved by adding to the column 5 ml of IFS pool [diluted 1:1 with 0.1 M PBS and filtered through 0.45  $\mu$ m acrodisc membrane filter (Gelman Sciences Inc., Ann. Arbor, MI, USA)]. Then, the column and the sera pool were left in an end over end mixer overnight at 4°C. The beads with the bound antigen-antibody complex were transferred into a BIO-RAD Glass Econo-column (length 20 cm, cross-sectional 0.39 cm<sup>2</sup>, maximum volume 8 ml). The unbound sera was drained, then the column was washed twice with 0.1 M PBS. The bounded antibodies were eluted by passing 5 bed-volumes of 0.2 M glycine (pH 2.8), through the column. The eluted antibodies were collected in 1 ml microfuge tubes each containing the neutralizing buffer [20  $\mu$ l 1 M phosphate buffer, pH 8 (SIGMA®)] to retain the reactivity of the collected antibodies. The pattern of the purified antibodies was analyzed

by SDS-PAGE. Then eluted IPAb fractions resulted from five runs on CNBr sepharose 4B-column were pooled.

Immunoreactivity of the immunopurified antibodies (IPAb): The reactivity of the IPAb eluted fractions was determined using EITB as described before. Each aliquot of the collected antibody fraction was tested against Fhwwh and the target antigen(s) strips and the most immunoreactive fractions were collected and pooled. Using EITB, the reactivity of the IPAb pool with Fhwwh and FhESP was detected in comparison with different sera pool [NHS, IFS and infected *S. mansoni* sera (ISS)].

Antibody-dependent cell-mediated cytotoxicity (ADCC): Peripheral blood mononuclear cells (PBMCs) was separated from erythrocytes by differential sedimentation in a dextran/saline solution. The effector cells neutrophils were purified by discontinuous density gradient centrifugation in Percoll (Polyvinylpyrrolidone-coated colloidal silica particles) (Pharmacia). Five ml of 80% Percoll were layered onto 5 ml of 60% Percoll [Percoll, 60% & 80% solutions in RPMI-1640]. Then 5 ml cell suspension were placed on the surface of the gradient followed by centrifugation at 500 x g (Sorvall<sup>®</sup>) for 30 min. at 4°C. The neutrophils were collected from the 60-80% interface (the medium-60% Percoll interface had monocytes, lymphocytes and red blood cells). Viability and count of cells were then determined by trypan blue exclusion test. Cells were then suspended at 10<sup>6</sup> cell/5ml RPMI-1640. Unless otherwise stated, all the previous steps took place under sterile conditions (Hudson and Hay, 1989). *F. hepatica* metacercariae (provided by Theodor Bilharz Research Institute, Egypt) was excysted as described by Duffus and Franks (1981) and Carmona *et al.* (1993). Aliquots of 100 µl/well (about 15 metacercariae/well) were transferred into 48 well cell culture plate (Costar, Costar-corporation, Cambridge, MA, USA) and incubated at 37°C in 5% CO<sub>2</sub> & 95% humid atmosphere in a CO<sub>2</sub> incubator (NuAire). After 3-6h. 70-80% of the flukes were excysted and seen actively moving. The adherence assay was performed (Duffus and Franks, 1980). One hundred µl of neutrophils (concentration 100x10<sup>3</sup> in culture media) and 100 µl of NHS (diluted 1:50), IFS (diluted 1:50) and IPAb (diluted 1:20, all dilutions were achieved in culture media) were added separately to previously

incubated NEJs in the culture plates. The plates were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> in CO<sub>2</sub> incubator. The plates were examined microscopically at different time intervals at magnifications 10x and 20x using Olympus inverted microscope (Germany). After 18h., bounded neutrophils No. was counted. Those flukes with more than 20 cells attached were considered positive (Carmona *et al.*, 1993). The viability & motility of the flukes were evaluated, parasites were scored dead when immotile and opaque in appearance.

## Results

The EITB pattern was nearly similar but not identical. The control sera or normal human subjects (NHS) showed no reactivity with Fhwwh bands in EITB. All fascioliasis sera specifically recognized with variable intensity Fhwwh 25-29 & 41 kDa bands. 82% of patients showed immunoreactivity with different bands between 19 kDa & 12 kDa. A strong immunoreactive band at 120 kDa was detected by 35% of patients and only 9% of the patients recognized a band of 63 kDa. The most immunoreactive bands are with low M<sub>r</sub>.

Table (1): Lymphocyte proliferation of *F. hepatica* infected and control subjects, cultured with Fhwwh, 12 kDa and 25-29 kDa antigens:

Antigen concentration	Sub	Fhwwh	12 kDa	25-29 kDa
2.5 µg/ml	i	1.63 ± 0.55	0.62 ± 0.12	1.92 ± 0.33*
	c	1.03 ± 0.03	0.56 ± 0.08	0.84 ± 0.11
	n	(6)	(6)	(6)
5 µg/ml	i	4.72 ± 0.78*	0.55 ± 0.18	2.32 ± 0.37*
	c	1.63 ± 0.58	0.76 ± 0.077	0.93 ± 0.11
	n	(6)	(7)	(6)
10 µg/ml	i	6.26 ± 0.73*	0.55 ± 0.14	2.00 ± 0.43*
	c	1.61 ± 0.50	0.30 ± 0.07	0.78 ± 0.07
	n	(6)	(6)	(7)

-Different concentrations (2.5, 5, 10 µg/ml) of each fraction used to stimulate infected and control lymphocytes. -Results expressed as E/C (E= mcpm of test well, C= mcpm of negative well) ± S.E. -(n) represents number of infected (i) and control (c) subjects. -"\*" Significant response as compared to controls (P < 0.05).



Resolving 10 mg/ml Fhwwh by 11% SDS-gel in 491- prep cell resulted fractions. An ascending ladder pattern of bands was indicative of good purification conditions. SDS-PAGE of 491-resolved fractions revealed protein fractions with  $M_r$  ranging from 12 kDa-40 kDa. *F. hepatica* specific immunoreactive fractions were evaluated by EITB using JFS (1:50). The 12 kDa and 25-29 kDa antigens were shown to be highly reactive with JFS. Each of the immunoreactive specific fractions were collected from eleven 491- runs, pooled, lyophilized, passed onto PD10 column for the removal of excess salts, followed by measurement of the protein content of each antigen alone as described before. It was found that loading 10 mg/ml Fhwwh in each run yield an average protein content of 150-250  $\mu\text{g/ml}$ . The estimated protein content of 12 kDa antigen was 1.617  $\mu\text{g}/\mu\text{l}$  and protein content of 25-29 kDa antigen was found to be 1.856  $\mu\text{g}/\mu\text{l}$ , after lyophilization of collected fractions from 491 runs (Fig. 2).

Concentrations (2.5, 5 & 10  $\mu\text{g/ml}$ ) of immunoreactive antigens (Fhwwh, 12 & 25-29 kDa polyantigens) were used to induce lymphoproliferative responses of peripheral blood mononuclear cells (PBMCs) of controls and *F. hepatica* infected subjects. The significance of differences between the cells lymphoproliferation of control subjects and the infected patients was assessed by Student's *t*-test. The results of representative controls and *F. hepatica*-infected subjects (Tab.1). Fhwwh was able to induce lymphoproliferative response at higher concentrations only (5, 10  $\mu\text{g/ml}$ ). The 12 kDa was unable to induce any significant lymphoproliferative responses in either group. Meanwhile, 25-29 kDa polypeptide antigen was able to induce a significant proliferation ( $P < 0.05$ ) of mono-nuclear cells (MNC) of infected subjects as compared to the control subject cells at different concentrations used (Tab.1) Thus, 25-29 KDa antigens were considered to be immunogenic fraction containing both T & B-cell epitopes for human fascioliasis, and therefore could be used in further studies. The protein pattern of Fhwwh in comparison Specificity and reactivity of the IPAb: The eluted IPAb fractions resulted from five runs on CNBr sepharose 4B-column were pooled. The SDS-PAGE pattern of the immunopurified eluted antibody (IPAb) revealed two bands at

$M_r$  55 kDa (heavy chain) and  $M_r$  25 kDa (light chain) indicating that pure antibody probe was obtained after immunoaffinity purification. The reactivity of the pooled IPAb probe was compared with normal human sera pool (NHS), infected *Fasciola* sera pool (IFS) and infected *S. mansoni* sera pool (ISS), against EITB strips of Fhwwh and FhESP (Fig. 5). All Sera were diluted (1:50) in the blocking buffer, except for IPAb where two dilutions were used (1:50 & 1:10 against strips of FhESP). As shown in Fig. (5A) NHS showed no reactivity with any of the Fhwwh bands (lane 2A). IFS showed reactivity with different bands of the Fhwwh pattern (lane 3A), the most intensely recognized polypeptides were at  $M_r$  72, 32-36, 29, 25, 17 & 12 kDa. ISS showed reactivity at  $M_r$  72 & 67 kDa (lane 4A), while IPAb showed reactivity at 29, 25 & 12 kDa (lane 5A). Similarly, when using FhESP strips (Fig. 5B), NHS & ISS faintly recognized a band in the range 25-24 kDa (lane 2B & lane 4B, respectively). IFS showed reactivity with 29, 25 & 17 kDa (lane 3B). IPAb showed reactivity with 29, 25 & 12 kDa (lane 5B), where those polypeptides become most intensely recognized when the 1:10 IPAb dilution was tested (lane 6B). with 25-29 kDa 491-purified antigens were analyzed by SDS-PAGE at conc. of 0.1  $\mu\text{g}/\mu\text{l}/\text{mm}$  (Fig. 4).

Specific antibody probe for this immunogenic fraction (25-29 kDa) was prepared using CNBr antigen-antibody affinity column. Lymphoproliferation test for target molecules: Newly excysted juvenile worms (NEJs) incubated with NHS neutrophils for 18h (conc.- $1001 \times 10^3$ ) were unable to adhere to parasites, as it lacks parasite antibodies (Fig. 6 A). Neutrophils adhered to juvenile flukes in large numbers presence of IFS (Fig. 6 B) and IPAb (Fig. 6 C & D), indicating adherence was dependent to antibody presence. The estimation of neutrophil adherence was subjective, since it was difficult to count accurately No. of attached cells on highly motile flukes. Flukes with more than 20 cells attached were considered positive (Carmona *et al.*, 1993).

## Discussion

In Egypt, the problem of human fascioliasis requires more efforts to avoid further spreading of the disease. Information on

the state of this disease in human and the nature of the protective human immune response directed against the parasite remains uncertain. The present study was designed as an attempt to characterize the most immunogenic antigens of *F. hepatica*, which are targets of host-protective immune responses and that could therefore be considered as vaccine candidates. To achieve this, fractionation of Fhwwh was carried out in an attempt to identify and characterize the specific *F. hepatica* antigen carrying major human T-cell and B-cell epitopes. The choice of the target antigen was governed by several parameters. First, the immunological characterization of the Fhwwh antigens using EITB technique showed that the 12 and 25-29 KDa antigens



Fig. (6 A-D): Effect of ADCC on NEJs flukes: Neutrophil attachment assay after 18 h. of neutrophils ( $100 \times 10^3$ ) incubation with NHS, IFS & IPAb (1:50, 1:50 & 1:20) at 37°C in 5% CO<sub>2</sub> and 95% humid atmosphere in CO<sub>2</sub> incubator, followed by microscopic examination at 20x magnification. In presence of NHS, neutrophils showed no attachment (A). In presence of IFS (B) and IPAb (C & D) neutrophils aggregated and adhered to juvenile flukes in large numbers with more significant adherence in case of IPAb (C & D).

were the most highly immunoreactive with the human fascioliasis sera. Second, the EITB pattern of Fhwwh 491-Prep cell eluted fractions showed that the highly immunoreactive antigens with fascioliasis sera pool were 12 & 25-29 KDa. Depending on the previous results, both 12 & 25-29 KDa antigens were purified and prepared for further studies. This result agree with Hillyer (1995; 1999) who defined the most immunogenic antigens as nFh12, a 12 KDa *Fasciola/Schistosoma* cross-reactive, cross-protective antigen related to the fatty acids binding proteins (FABPs). Another antigens showing intense humoral responses were defined at the zone 27-30 KD and could be considered as GST and cathepsin proteases.

On the other hand, the proliferative responses of peripheral blood mononuclear cells (PBMC) of infected subjects showed that, the 25-29 kDa antigen(s) (2.5, 5 & 10 µg/ml) induced significant lymphoproliferative response of PBMC. Based on this results, the 25-29 kDa antigen(s) was considered immunogenic containing major human T-cell & B-cell epitopes. A human polyclonal mono-specific immunopurified antibody against 25-29 kDa antigen(s) was prepared. In the light of results, it should be noted that when IPAb was tested by EITB against Fhwwh and FhESP, the patterns obtained were somewhat similar with 29-25 KDa & 12 KDa as the most prominent bands detected. This could be explained on the basis of a cross-reactivity between those antigens in both Fhwwh and FhESP, or the antigens might be immunologically related, where both antigens might share a specific epitope recognized by the IPAb or the IPAb contained antibodies directed to different distributed epitopes.

The IPAb involvement in ADCC against NEJs was also evaluated. Previous studies showed that, ADCC has a cytotoxic response that is mediated by cells and dependent on the presence of antibody. This response plays a predominant role in killing invading parasite larvae of helminths (Capron *et al.*, 1982). Using this assay, it was evident that human neutrophils were more effective at adhering to the IPAb antibody-coated flukes (NEJs), followed by those coated with infected *Fasciola* subjects antibodies or normal human subjects antibodies. This attachment persisted for almost 18h without affecting the

viability or the motility of the parasite. Thus, it could be suggested that in the *in vitro* system, fluke rapid turnover and excretion of the outer glycocalyx opposes the intimate attachment of neutrophils to the parasite, which is a prerequisite for any cell mediated damage to occur.

In conclusion, the study identified and purified the most immunogenic *F. hepatica* target antigen(s). The isolated antigens were used to prepare an IPAb probe, whose participation in potentially lethal ADCC reaction was proved. The usage of the IPAb in further molecular and histological characterization of the target antigen(s) is ongoing and will be published later..

### Acknowledgements

The authors thanks the members of the Egyptian Reference Diagnostic Center (ERDC) at Egyptian Organization for Biological Products and Vaccines (VACSERA) for the kind help during the practical work.

### References

- Abdel-Rahman, S.; O'Reilly, K.L. and Malone, J.B. (1999):** Biochemical characterization and localization of *Fasciola hepatica* 26-28 kDa diagnostic coproantigen. *Parasite Immunol.*, 21(6):279-286.
- Allam, A.F.; El-Agamy, S.I. and Helmy, M.H. (2002):** Molecular and immunological characterisation of *Fasciola* species. *Br. J. Biomed. Sci.*, 59(4):191-195.
- Arjona, R.; Riancho, J.A.; Salesa, R. and Gonzalez-Macias, J. (1995):** Fascioliasis in developed countries: a review of classic and aberrant forms of the disease. *Medicine*, 74: 13-23.
- Bradford, M.M. (1976):** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254.
- Capron, A.; Dessaint, J.P. Haque, A. and Capron, M. (1982):** Antibody-dependent cell mediated cytotoxicity against parasites. *Progr. Allergy*, 31: 234-267.
- Carmona, C.; Dowd, A.J.; Smith, A.M. and Dalton, J.P. (1993):** Cathepsin L proteinase secreted by *Fasciola hepatica* in

*vitro* prevents antibody-mediated eosinophil attachment to newly excysted juveniles. Mol. Biochem. Parasitol., 62: 9-18.

**Duffus, W.P.H. and Franks, D. (1980):** *In vitro* effect of immune serum and bovine granulocytes on juvenile *Fasciola hepatica*. Clin. Exp. Immunol., 41: 430-440.

**Duffus, W.P.H. and Franks, D. (1981):** The interaction *in vitro* between bovine immunoglobulin and juvenile *Fasciola hepatica*. Parasitology, 82: 1-10.

**Farrant, J.; Clark, J.C.; Lee, H.; Knight, S.C. and O'Brien, J. (1980):** Conditions for measuring DNA synthesis in PHA stimulated human lymphocytes in 20 µl hanging drops with various cell concentrations and periods of culture. J. Immunol. Methods, 33: 301-312.

**Haseeb, A.N.; El-Shazly, A.M.; Arafa, M.A. and Morsy, A. T.A. (2003):** Evaluation of excretory/secretory *Fasciola* (Fhes) antigen in diagnosis of human fascioliasis. J. Egypt. Soc. Parasitol., 33(1):123-138.

**Hillyer, G.V. (1995):** Comparison of purified 12 kDa and recombinant 15kDa *Fasciola hepatica* antigens related to *Schistosoma mansoni* fatty acid binding protein. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, 90(2): 249-253.

**Hillyer, G.V. (1999):** Immunodiagnosis of human and animal fascioliasis. In: Fasciolosis. Dalton J.P. (ed.). CABI, Wallingford, UK and New York.

**Hillyer, G.V. and Apt, W. (1997):** Food-born trematode infections in the Americas. Parasitol. Today, 13: 87-88.

**Hillyer, G.V. and Soler de Galanes, M. (1988):** Identification of a 17-Kilodalton *Fasciola hepatica* immunodiagnostic antigen by the enzyme-Linked immunoelectrotransfer blot technique. J. Clin. Microbiol., 26(10): 2048-2053.

**Hillyer, G.V.; del Llano de Diaz, A. and Reyes, C.N. (1977):** *Schistosoma mansoni*: Acquired immunity in mice and hamsters using antigens of *Fasciola hepatica*. Exp. Parasitol., 42: 348-355.

**Hudson, L. and Hay, F. (1989):** Immunological techniques in clinical medicine. In: Practical Immunology. Blackwell Scientific Publications, Oxford London.

- Korach, S. and Benex, J. (1966):** A lipoprotein antigen in *Fasciola hepatica*. I. Isolation, physical and chemical data. *Exp. Parasitol.*, 19: 199-205.
- Makowski, G.S. and Ramsby, M. L. (1998):** Protein molecular weight determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis. In: Protein Structure "A Practical Approach". Creighton T.E. (ed). European Molecular Biology Laboratory, Heidelberg, Germany.
- Marin, M.S.; Prieto, M.; Martin, M.J.; Casais, R.; Boga, J.A. and Parra, F. (1992):** Encoding a gut antigen protein bearing repetitive sequences. *Mol. Biochem. Parasitol.*, 55: 155-166.
- Morrissey, J.H. (1981):** Silver stain for protein in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Anal. Biochem.*, 117: 307- 310.
- Pfister, K.; Turner, K.; Currie, A.; Hall, E. and Jarrett, E.E. (1983):** IgE production in fascioliasis. *Parasite Immunol.*, 5: 587-593.
- Piacenza, L.; Acosta, D.; Basmadjian, I.; Dalton, J.P. and Carmona, C. (1999):** Vaccination with cathepsin L proteinases & with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. *Infect. Immun.*, 67(4): 1954-1961.
- Poitou, I.; Baeza, E. and Boulard, C. (1993):** Kinetic responses of parasite-specific antibody isotypes, blood leucocyte pattern and lymphocyte subset in rats during primary infestation with *Fasciola hepatica*. *Vet. Parasitol.*, 49: 179-190.
- Saba, R.; Korkmaz, M.; Inan, D.; Mamikoglu, L.; Turhan, O.; Gunseren, F.; Cevikol, C. and Kabaalioglu, A. (2004):** Human fascioliasis. *Clin. Microbiol. Infect.*, 10(5):385-387.
- Sampaio Silva, M.L.; Correia Da Costa, J.M.; Da Costa, A. M.; Pires, M.A.; Lopes, S.A.; Castro, A.M. and Monjour, L. (1996):** Antigenic components of excretory-secretory products of adult *Fasciola hepatica* recognized in human infections. *Am. J. Trop. Med. Hyg.*, 54(2): 146-148.
- Santiago, N. and Hillyer, G.V. (1986):** Isolation of potential serodiagnostic *F. hepatica* antigens by electroelution from polyacrylamid gels. *Am. J. Trop. Med. Hyg.*, 35(6):1210-1217.
- Santiago, N. and Hillyer, G.V. (1988):** Antibody profiles by EITB and ELISA of cattle and sheep infected with *Fasciola hepatica*. *J. Parasitol.*, 74(5): 810-818.

- Tsang, V.C.W.; Peralta, J.M. and Simons, A.R. (1983):** Enzyme-linked immunoelectrotransfer techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. In: Methods in enzymology. Langone, J.J. and Van Vunakis (eds). Vol. 92. Academic Press, New York.
- Tsang, V.C.W. ; Bers, G.E. and Hancock, K. (1985):** Enzyme-linked immunoelectrotransfer blot (EITB). In: Enzyme-mediated Immunoassay. Ngo, T.T. & Jenhoff, H.M. (eds), Plenum Press, New York.
- Van Milligen, F.J.; Cornelissen. J.B.W.J.; Hendriks, I.M.; Gaasenbeek, C.P.H. and Bokhout, B.A. (1998):** Protection to *Fasciola hepatica* in the gut mucosa of immature rats is associated with infiltrates of eosinophils, IgG1 and IgG2a antibodies against the parasites. Parasite Immunol., 20: 285-292.
- WHO, (1995):** Control of foodborne trematode infections. WHO Techn. Rep. Ser., No. 849.
- Zurita, M.; Bieber, D.; Ringold, G. and Mansour, T.E. (1987):** Cloning and characterization of a female genital complex cDNA from the liver fluke *Fasciola hepatica*. Proc. Natl. Acad. Sci. USA, 84: 2340-2344.

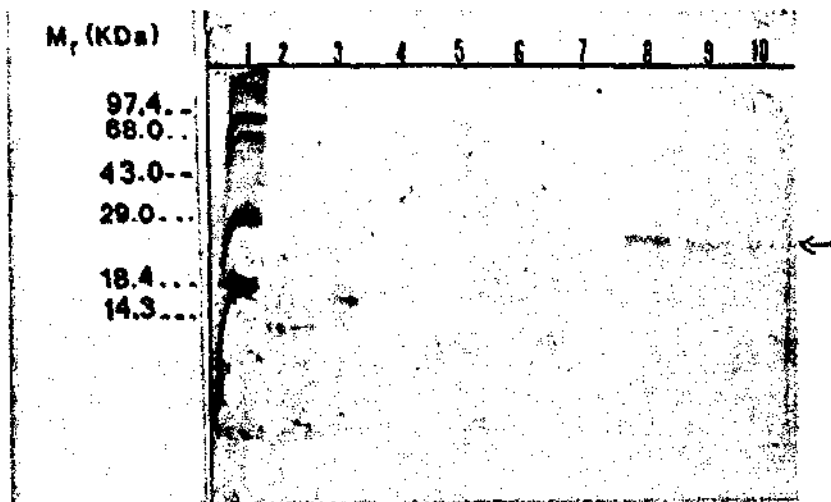
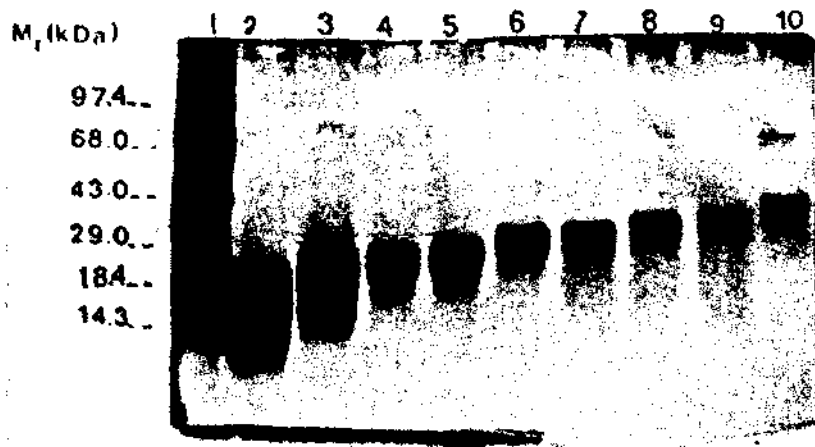
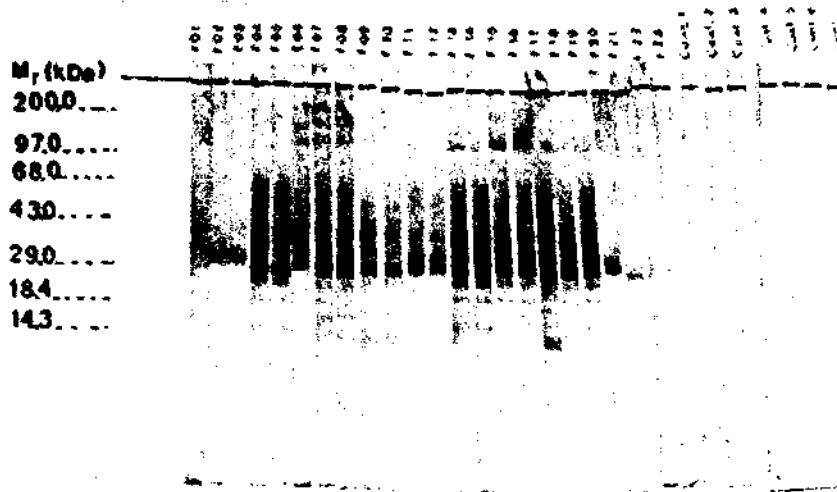
### Explantation of Figures

Fig. 1: Reactivity of sera from *F. hepatica* infected subjects with Fhwwh pattern using EITB. Fhwwh proteins (0.1 µg/µl/mm) separated by 5-22.5% SDS-PAGE, transferred onto one NC sheet, cut into strips. NC strips originated from same blot with IFS (F01-F23) and NHS (Cont.1- Cont.7), dilution 1:50, reacted with NC strips. Transferred  $M_r$  markers on right.

Fig. 2: Silver stain of ladder shape pattern of Fhwwh antigen fractions separated by 491-prep-cell. Electrophoretic conditions: slab gradient 5-22.5% SDS-PAGE, Antigen: Fhwwh 491-prep cell resolved fractions, lane 2-10 Load: 0.1 µg/µl/mm of antigen/lane; Sample buffer: reducing; Current: 15 mAmp/slab; Staining: silver staining. Positions of transfered  $M_r$  markers on right side.

Fig. 3: Immunoreactivity of 491-prep cell fractions (7-30 KDa) in EITB. Fhwwh separated by 491-prep cell (low  $M_r$  separated fractions 7- 40 KDa) resolved by SDS-PAGE (5-22.5%) slab gels, blotted onto NC sheets, reacted with IFS pool (1:50). Arrow on left side indicate positions of most immunoreactive antigen.





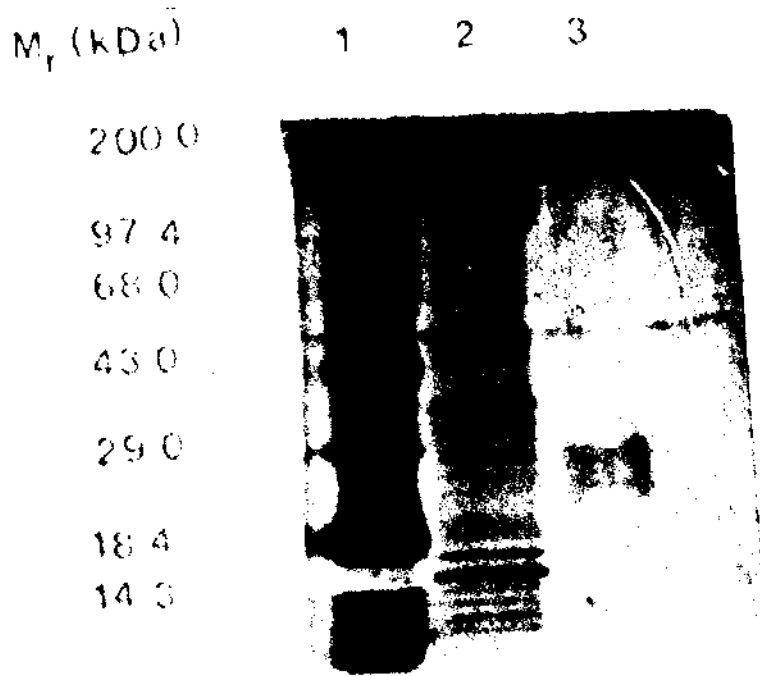


Fig. 4. Silver stain of Fhwvh and 25-29 KDa purified antigens. Electrophoretic conditions: slab, gradient 5-22.5% SDS-PAGE; Samples, Fhwvh and 491-purified antigen, lanes 2,3 respectively. Load: 0.1 µg/µl/mm protein/lane; Sample buffer: non-reducing; Current: 7.5 mAmp/slab gel; Staining: silver nitrate. Analysis of Fhwvh revealed about 20 major bands with  $M_r$  ranging from 7-200 KDa, with transferred marker on right side.

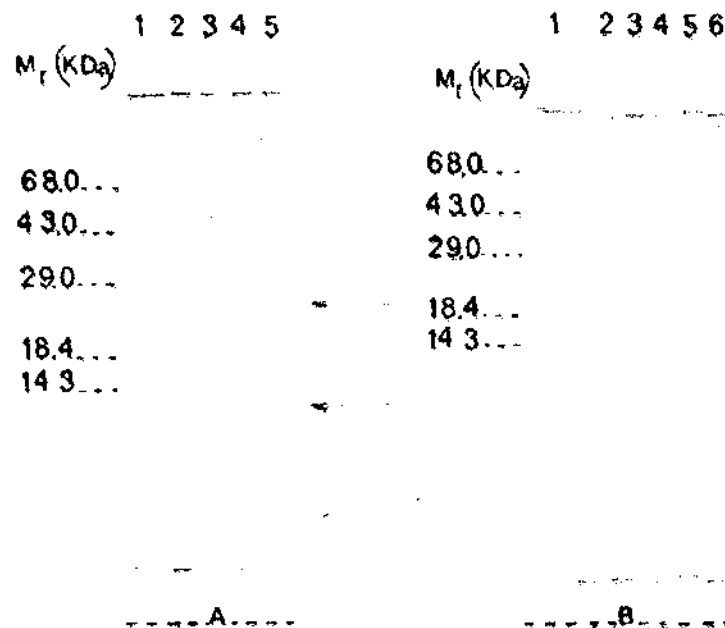


Fig. 5: Comparison of reactivity of NHS, IFS, ISS and IPAb with strips of Fhwvh and FhESP. Fhwvh and FhESP individually separated by 5-22.5% SDS-PAGE, transferred onto NC sheets and reacted with NHS, IFS, ISS and IPAb respectively. NC strips of each antigen originated from one slab gel with a single large sample trough. Fhwvh strips (A): reaction with 1:50 diluted NHS (lane 2A), IFS (lane 3A), ISS (lane 4A) & IPAb (lane 5A). FhESP strips (B) reaction with NHS (1:50) (lane 2B), IFS (1:50) (lane 3B), ISS (1:50) (lane 4B) & IPAb (1:50) (lane 5B) & (1:10) (lane 6B). Arrows on the left side shows the most immunoreactive bands with IPAb (lane 5A; 5&6B). Transferred  $M_r$  marker lane 1A and lane 1B on right side.