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Fasciola gigantica Excretory/secretory Antigens as Possible Vaccine Candidates

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A variety of antigens are secreted and excreted by parasites present in the blood, faeces, urine and other fluids of the infected host. These antigens have potential for use in immunodiagnosis and vaccine development. In an attempt to develop a suitable vaccine against *F. gigantica* infection, two antigens were isolated and purified from excretory/secretory (E/S) products as cysteine protease and fatty acid binding proteins (CP and FABP) of the parasite by immunoaffinity chromatography. Parasitological and immuno-logical parameters were standardized using the sera from experimentally non-infected (group A) infected (group B), immunized with CP (group C), immunized with CP and infected (group D), immunized with FABP (group E) and immunized with FABP and infected (group F). The mean worm burdens and bile egg count after challenge were reduced significantly by 37.7 and 55.5%, respectively in rabbits vaccinated with CP. In contrast, low significant reduction in worm burdens and bile egg count were observed in rabbits immunized with FABP after challenge (23.5 and 35.7%, respectively). All *F. gigantica* infected rabbits showed an increased Igs and cytokines levels. On the other hand, immunization of rabbits with CP or FABP induced a significant expression of humoral antibodies (IgM, total IgG, IgG1, IgG2 and IgG4) and cytokines (IL-6, IL-10, IL-12 and TNF-α) with higher level in case of CP than FABP. From this study, we can deduce that *F. gigantica* CP is a relevant candidate for vaccination against fascioliasis, while the level of protection used by FABP may not appear sufficient enough to protect these ruminants against the deleterious effects of *Fasciola* infection.

Key words: *F. gigantica*, cystein protease, fatty acid binding proteins, immunoglobulins, cytokines, vaccine

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INTRODUCTION

Grass-grazing animals are often infected with fascioliasis which affects their growth and productivity due to the resistance of the flukes to traditional drugs it is of vital importance to develop a substitute mode to overcome such an infection. Vaccination would be a viable alternative. Till now, no effective vaccine against liver fluke infection has been commercialized (Marcos *et al*., 2009).

Ex vivo F. hepatica and *F. gigantica* worms release substantial amounts of excretory-secretory antigen (E/S) (Spithill *et al*., 1999). The use of *Fasciola* worms-derived E/S, namely Cysteine Protease (CP), for serodiagnosis implies that naturally infected cattle and sheep were shown to mount vigorous cellular and humoral immune responses to E/S during *Fasciola gigantica* (*F. gigantica)* natural infection. However, few reports are concerned with whether the E/S-mediated immune responses are associated with protection against fascioliasis (El-Ridi *et al*., 2007). Two dimensional gel electrophoresis analysis indicated that *F. hepatica* release approximately 60 proteins, of which 29 consist essentially of cathepsin L proteases (CL), superoxide dismutase, thioredoxin peroxidase, glutathione-S-transferase (GST) and Fatty Acid Binding Proteins (FABP) (Jefferies *et al*., 2001).

Rapid and accurate diagnosis of the infection is of primary importance for starting immediate chemotherapy. Complete cure is reported following treatment with trichlorobendazole, provided early and accurate diagnosis of the infection (Spithill *et al*., 1999). Diagnosis depended mostly on fecal examination for the detection of eggs taking into consideration that fasciola worms excrete eggs at intervals, developing on that technique will not be accurate. This renders stool parasitological examination a notably difficult, insensitive and unreliable method for diagnosis of veterinary fascioliasis. Besides that, this technique does not allow the detection of early-stage prepatent infection which lasts approximately three month (Dixit *et al*., 2002). Diagnosis by antibodies detection to specific worm antigens would be more through and definitive.

Despite that chemotherapy with triclabendazole is effective in treatment, it does not prevent reinfection. Therefore, animals in endemic countries must be continuously treated with the drug. This approach is prohibitively expensive for developing countries and furthermore, promotes the threat of drug resistance. Controlling fascioliasis by vaccination, rather than chemotherapy, would be a cheaper, more efficient and reliable long-term solution for the prevention of the infection and eradication of its transmission. To date, there are few defined antigens as vaccines for *F. gigantica* or *F. hepatica* (Spithill *et al*., 1999). Vaccine trials were conducted in cattle and sheep to evaluate the efficacy of *F. gigantica*-derived native GST, paramyosin, FABP and CL against challenge infection with metacercariae of *F. hepatica* or *F. gigantic* (Spithill *et al*., 1999). CL proteinase generated significant levels of protection against *F. hepatica* in sheep (Dalton *et al*., 2003).

There are numerous studies on the use of purified native or recombinant molecules as candidate vaccines (Hillyer, 2005; McManus and Dalton, 2006). These include CL, a family of proteases involved in activities such as immune evasion (Carmona *et al*., 1993; Smith *et al*., 1993), invasion of tissues (Berasain *et al*., 1997), nutrition (Smith *et al*., 1993) and egg output (Dalton *et al*., 1996). These activities may interfere with their protective effect; thus, it would be desirable to isolate the immunizing motifs for vaccination purposes. Moreover, while native CL could be purified in sufficient quantities for experimental trials, large-scale production requires a cheaper system, i.e., competitive with current drug treatments (Dalton *et al*., 2003).

Native or recombinant FABP (rFABP) from *F. hepatica* has important immunoprophylactic interest against fascioliasis (Hillyer, 2005). The native Fh12 FABP with Freund's adjuvant has demonstrated protections of 55% in calves (Hillyer *et al*., 1987), 78% in mice (Hillyer, 2005) and 40% in rabbits (Muro *et al*., 1997) against *F. hepatica*. An anti-fecundity effect has been observed in sheep (Ramajo *et al*., 2001).

This work aimed to study the humoral and cellular immune responses to different *F. gigantica* antigens isolated from adult worm E/S product (CP and FABP) during the parasite invasion.

MATERIALS AND METHODS

Animals: Newzealand white male rabbits, (1.5 kg wt., 1.5 months age), were obtained and housed in the Schistosome Biological Supply Program, Theodore Bilharz Research Institute (SBSP, TBRI), Giza, Egypt. They were kept under standard laboratory care (Nessim *et al*., 2000). Rabbits were examined before the experiments to prove free from *Fasciola* and other parasitic infection.

Parasites: Adult fresh *F. gigantica* worms were collected out of biliary tracts and gall bladders of naturally infected bovine livers. The live intact worms were washed several times with Phosphate-buffered Saline (PBS) (pH 7.4) to remove the host blood, bile and contaminating

microorganisms (Maggioli *et al*., 2004). Metacercariae of *F. gigantica* were purchased from SBSP-TBRI.

Preparation of antigens

***Fasciola* worm antigen preparation (E/S):** Adult fresh *F. gigantica* worms were removed from the bile ducts of the livers and washed 6 times with cold 0.01 M PBS containing 125 mM NaCl for 1 h. The worms were incubated for 16 h at 37°C in RPMI 1640 medium (pH 7.4). Following incubation, the medium removed and centrifuged at 3000 rpm for 30 min and the supernatant containing E/S products was collected and stored at -20°C as aliquots until used (Maleewong *et al*., 1999). The protein content of E/S antigen was measured by the Bio Rad protein assay kit (Bradford, 1976).

Purification of *F. gigantica* fatty acid binding protein (FABP): FABP antigen was purified from the crude extract of adult *F. gigantica* worms by ion exchange chromatography on diethyl amino ethyl Sephadex A-50 (DEAE-Sephadex A-50). Sephadex A-50 powder (1 g) (Amersham Bioscience, Uppsala, Sweden) was swelled in about 200 mL of 0.5 M Tris-HCI buffer (pH 7). After swelling, beads were washed extensively with 10 mM Tris HCl buffer (pH 6.5). The slurry (75%) was poured in 30×2.5 cm column (Bio-Rad) in one continuous motion to minimize air bubbles.

Following beads settling, the column was equilibrated with 3 bed vol. (10 mM Tris HCl) and the approximate column binding capacity was determined. The sample was dialyzed versus the eluting buffer and its protein content was calculated. The beads were washed by 4 bed vol. eluting buffer. The substance of interest (FABP) is washed out of the gel (Timanova *et al*., 1999), 1 mL fractions collected under gravity and absorbance at 280 nm of each fraction was measured.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): The crude extract and protein fractions were characterized using discontinuous SDS-PAGE in 12% slab gels (Bio-Rad) under reducing conditions as described by Laemmli (1970) and Takacs (1979).

Purification of *F. gigantica* cysteine proteinase: E/S products were concentrated using an Amicon 8400 ultrafiltration unit with membrane (3000 kDa cut-off). The sample applied to a 120 mL Sepharsyl S200 HR column (gel filtration chromatography) equilibrated in 0.1 M Tris-HCl. Fractions of 5 mL were collected. The column elute was monitored at 280 nm. Fractions were analyzed for enzymatic activity (Smith *et al*., 1993).

Those fractions containing the group 1 CP (CL1 and CL2), described by Dalton and Heffernan (1989), were pooled and applied to a 50 mL QAE Sephadex column equilibrated in 0.1 M Tris-Hcl. The unbound fraction (150 mL) was collected and concentrated in an Amicon ultrafiltration unite to a vol. of 10 mL, was dialyzed against ultra-pure water and freeze dried.

Experimental design

Immunization and challenge infection: Rabbits were divided into six groups with 8 rabbits in each group.

Group A: Normal control rabbits

Group B: Infected control rabbits that were infected orally with 25 *F. gigantica* metacercariae

Group C, E: Immunized groups. Rabbits were immunized with 1 mg of CP or FABP, respectively, intramuscularly (i.m) with equal vol. of Complete Freund's Adjuvant (CFA) as primary immunization on day 0. Booster doses, 1 mL of 0.5 mg CP or FABP mixed with an equal vol. of Incomplete Freund's Adjuvant (IFA), were administered after 2, 3 and 4 week after the 1st dose as 2nd, 3rd and 4th immunizations

Group D, F: Infected immunized groups. Rabbits were immunized with the same schedule of the group C. Each of these animals was challenged orally with 25 *F. gigantica* metacercariae (Muro *et al*., 1997) after 2 week of 4th immunization

Blood samples were collected weekly along the immunization program and at 2 week interval Post Infection (PI) and the sera were prepared and examined by ELISA for cross reactivity detection. When the titer was detectable, rabbits were sacrificed and antisera were collected and stored at -20°C till used.

Parasitological criteria

Fluke size and weight: All rabbits were euthanized in the 12 week PI. Flukes were collected and recorded for their net weight, length and width. Percent Reduction (PR) was calculated comparing the number of worms recovered from different groups with the infected control, by the formula:

$$PR = \frac{\text{Mean worm recovery from control group} - \text{Mean worm recovery from test group}}{\text{Mean worm recovery from control group}} \times 100$$

Bile egg count: Bile collected from infected control and infected immunized rabbits were cleared by repeated sedimentation/decantation of the eggs to calculate mean total egg count. The PR was calculated using the formula:

$$PR = \frac{\text{Mean No. of eggs in control group} - \text{Mean No. of eggs in test group}}{\text{Mean No. of eggs in control group}} \times 100$$

Immunological parameters

Humoral immune response

Assessment of anti-*Fasciola* total IgG and IgG4 by ELISA: ELISA test was assessed according to modifications of Nilsson (1990) and Venkatesan and Wakelin (1993).

Cellular immune response

Peripheral blood mononuclear cells (PBMNCs) proliferation in response to *F. gigantica* antigens: Heparinized blood samples were collected from infected sheep. PBMNCs was separated over Ficol Hepaque (Seramid Biochrom, Berlin) and washed 4 times with PBS (Chitko-McKown *et al*., 2004).

The PBMNCs layer was spreaded on cytoslide microscope slides, by using a cytospin® 3, cell preparation system. Cells were resuspended "3×105" in 500 μL medium and loaded into a cytofunnel® (Shandon Lipshaw, Pittsburgh, PA) to permit adherence of the cell onto microscope cytoslide then immunoperoxidase technique was applied.

Statistical analysis: Data were expressed as Mean±SD and analyzed statistically using the student's t-test. The data were considered significant if p values were equal to or less than 0.05.

RESULTS

Purification of CP from E/S products: *F. gigantica* purified E/S products using DEAE-Sephadex A-50 ion exchange column chromatography, were analyzed by 12.5% SDS-PAGE under reducing conditions. The partially purified CP antigen (three bands were obtained at 55, 45 and 27.5 kDa, respectively) was passed through DEAE sepharsyl G-200 gel filtration column chromatography, then was analyzed by 12.5% SDS-PAGE under reducing condition showed only CP band at 27.5 kDa with final protein content was 11.6 mg mL^{-1} (Fig. 1).

Purification of FABP from E/S products: E/S antigens of adult *F. gigantica* were analyzed with DEAE-Sephadex G-200 ion exchange column chromatography. The eluted proteins from the gel filtration column chromatography were analyzed by 12.5% SDS-PAGE under reducing condition showed three bands at 95, 55 and 14 kDa including FABP protein. The partially purified FABP antigen was passed through DEAE Sepharsyl HR-100 gel

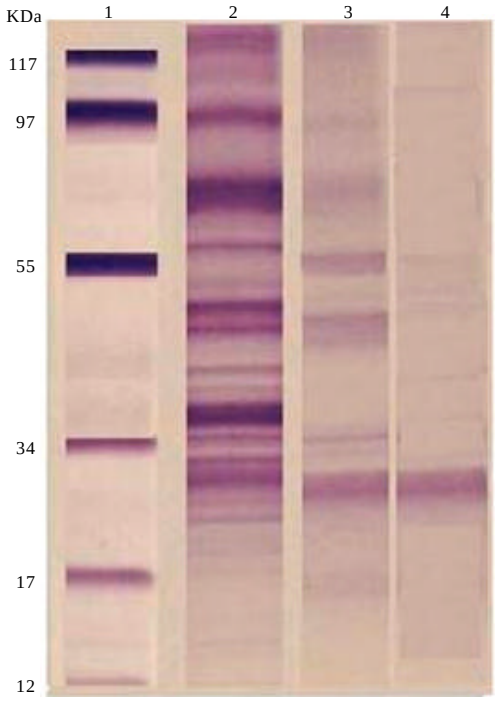

Fig. 1: SDS-PAGE 12% analysis; Lane 1: Standard proteins, Lane 2: Crude extracts of *F. gigantica* adult worms, Lane 3: Partially purified adult *F. gigantica* worms CP antigen eluted from DEAE Sephadex A-50 column, Lane 4: Adult *F. gigantica* worms CP eluted from sepharsyl G-200 column. The gel was stained for protein with Coomassie blue

filtration column chromatography. The eluted proteins were analyzed by 12.5% SDS-PAGE under reducing condition showed only FABP band at 14 kDa with final protein content was 3.8 mg mL^{-1} (Fig. 2).

Parasitological criteria

Effect of CP and FABP on the mean number of total worm burden and bile ova count: Immunization of infected rabbits with CP (group D) or FABP (group F) stimulated a significant ($p<0.01$) decrease (11.1±2.9 and 13±3.3, respectively) in mean total number of worm burden with a Percentage Reduction (PR) of (34.7 and 23.5%, respectively). Also, CP and FABP immunized infected rabbits (group D and F) recorded 4669±189 and 6755±145 as the mean total number of *F. gigantica* egg load showing a very highly significant decrease ($p<0.001$) than the infected control (group B) (10500±213), with PR: 35.5 and 55.7% (Table 1).

Table 1: The effect of CP and FABP on the worm burden and bile egg count in rabbit before and after infection with 25 *F. gigantica* metacercariae

Animal group	Worm burden (Mean±SD)	Reduction (PR) (%)	Mean bile egg count (Mean±S.D)	Reduction (PR) (%)
A: Normal control	-	-	-	-
B: Infected control	17±4.2	-	10500±213	-
C: Immunized with CP	-	-	-	-
D: Immunized with CP and infected	11.1±2.9**	34.7	4669±189***	55.5
E: Immunized with FABP	-	-	-	-
F: Immunized with FABP and infected	13±3.3*	23.5	6755±145**	35.7

Significant difference p<0.05*, Highly significant difference p<0.01**, Very highly significant difference p<0.001***, CP: Cystein proteinase, FABP: Fatty acid binding protein, PR: Percentage reduction, Data are represented as Mean±SD

Table 2: Effect of CP and FABP immunization on serum level of IgM, total IgG, IgG1, IgG2 and IgG4 levels in rabbits before and after infection with 25 *F. gigantica* metacercariae

Animal group	IgM	Total IgG	IgG1	IgG2	IgG4
A: Normal control	0.24±0.013	0.29±0.023	0.31±0.027	0.22±0.020	0.33±0.01
B: Infected control	0.82±0.022	1.31±0.012	0.51±0.012	0.62±0.016	0.94±0.026
C: Immunized with CP	0.78±0.012***	1.01±0.017***	0.69±0.015**	0.73±0.024***	0.88±0.027***
D: Immunized with CP and infected	0.96±0.024Δ	1.70±0.022ΔΔ	0.87±0.021ΔΔ	0.53±0.019	1.21±0.021ΔΔ
E: Immunized with FABP	0.65±0.02***	0.83±0.021***	0.34±0.021	0.33±0.011*	0.74±0.011**
F: Immunized with FABP and infected	0.83±0.02	1.50±0.022Δ	0.65±0.022Δ	0.53±0.013	1.13±0.031ΔΔ

Significant difference p<0.05 (*, Δ), *Significant difference between groups A, C and E, Highly significant difference p<0.01 (**, ΔΔ), Δ: Significant difference between groups B, D and F, Very highly significant difference p<0.001 (***, ΔΔΔ), Data are represented as Mean±SD

Immunological criteria

Effect of CP and FABP on IgM and total IgG levels and their isotypes (IgG1, IgG2 and IgG4) in different studied groups: The data illustrated in Table 2 showed levels of IgM, IgG and its isotypes (IgG1, IgG2 and IgG4) of anti-CP and anti-FABP injected in normal and infected rabbits that were assayed using indirect ELISA.

Serum IgM level: There is a significant elevation (p<0.001) of IgM level in infected control (group B) (0.82±0.022), CP or FABP immunized groups (group C and E) (0.78±0.012 and 0.65±0.02, respectively), in comparison to normal control group (group A) (0.24±0.013). Rabbits immunized with CP before infection (group D) recorded the highest significant (p<0.05) level (0.96±0.024) while FABP immunization before infection (group E) recorded the same level of infected control group (0.83±0.02) (Table 2).

Serum total IgG level: As depicted in Table 2, infection itself causes an evident increase in the level of total IgG (1.31±0.012) (group B) when compared to normal control rabbits (group A) (0.29±0.023). Immunization of purified E/S antigens (CP or FABP) before infection (Group C and E) caused an extraordinary significant (p<0.001) increase on total IgG level than normal control group (1.01±0.017 and 0.83±0.021, respectively). On the other hand, immunization with CP or FABP preinfection increased the IgG level in serum to 1.70±0.022 and 1.50±0.022, respectively. It is noticed that CP induced higher IgG level more than FABP antigen (Table 2).

Serum IgG1 level: The infection of rabbits (group B) induced IgG1 level elevation (0.51±0.012) compared to

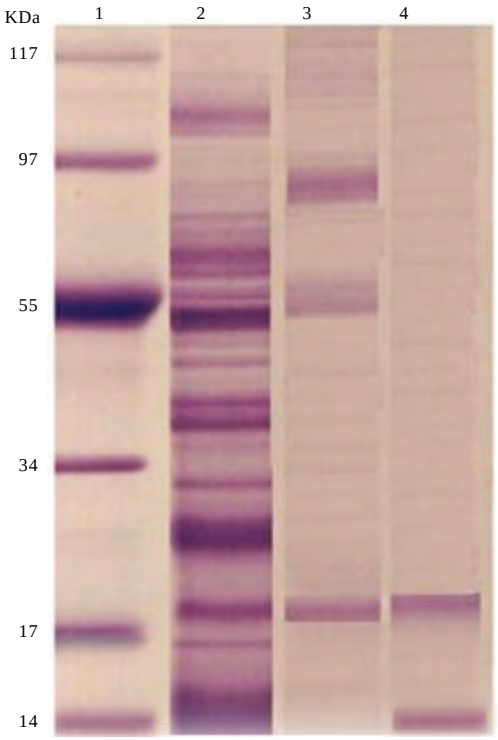

Fig. 2: SDS-PAGE12% analysis; Lane 1: Standard proteins, Lane 2: Crude extracts, Lane 3: Partially purified adult *F. gigantica* worms FABP antigen eluted from DEAE Sephadex A-50 column, Lane 4: Adult *F. gigantica* worms Purified FAPB eluted from sepharsyl HR-100 column. The gel was stained for protein with Coomassie blue

normal rabbits (group A) (0.31±0.027). Immunization with CP (group C) had an extraordinary effect on IgG1, significantly ($p<0.01$) increasing its level higher than the rabbits immunized with FABP (group E) (0.69±0.015 and 0.34±0.021, respectively). When the immunized rabbits with CP were infected (group D), IgG1 level recorded 0.87±0.021, with a significant elevation ($p<0.01$). Also, FABP immunized-infected rabbits (group F) induced a significant elevation ($p<0.05$) in IgG1 level (0.65±0.022) relative to infected untreated rabbits (Table 2).

Serum IgG2 level: Data indicated that infection caused a significant elevation in the IgG2 level (0.62±0.016) in comparison to normal control group (0.22±0.020). The IgG2 level in sera of rabbits immunized with CP (group C) (0.73±0.024) showed a very highly significant elevation ($p<0.001$) relative to normal rabbits (group A). Also, FABP immunization recorded a significant difference IgG2 level (group E) ($p<0.05$) (0.33±0.011). Groups D and F recorded 0.53±0.019 and 0.53±0.013, respectively as serum IgG2 level. On the other hand, infection after immunization with CP or FABP has no effect on the IgG2 level than the only infected group (Table 2).

Serum IgG4 level: The level of IgG4 after infection recorded a highly significant elevation ($p<0.05$) (0.94±0.026) in comparison to normal control group (0.33±0.01). Immunization of rabbits with CP (group C) or FABP (group E) recorded 0.88±0.027 and 0.74±0.011, respectively, as a highly significant increase ($p<0.001$) in comparison to the normal rabbits group. In case of groups D and F, in which rabbits were infected post immunization, the IgG4 levels recorded a highly significant ($p<0.01$) jump recording the highest level between all groups (1.21±0.021 and 1.13±0.031, respectively) in comparison to the normal or infected control groups (Table 2).

All data of both CP immunized groups, uninfected or infected (group C and D) indicated the higher immunostimulatory effect of CP than FABP antigens.

Effect of CP and FABP immunization on Th1 and Th2 cytokines level in different studied groups

Serum IL-6 level: The infection itself induced an elevation in IL-6 level (group B) recording 114.82±12.23, relative to normal rabbits (group A) (13.26±2.19). Immunization with CP (group C) or FABP (group E) antigens only increased significantly ($p<0.001$) the IL-6 level to 40.41±9.29 and 90.33±8.24, respectively. While immunization was followed by infection (group D and F), the serum IL-6 level recorded a non significant elevation (48.89±7.33 and 66.80±9.31, respectively), but lower than infected group (group B) (Table 3).

Serum IL-10 level: Relative to normal control rabbits (group A), the IL-10 level showed a highly significant increase ($p<0.001$) after CP or FABP treatment (group C and E) (82.31±8.89-270.71±14.21 and 253.65±21.25, respectively). Data showed an increase in IL-10 level with infection (group B) (487±16.44). Pre-immunization of infected rabbits with CP or FABP (group D and F) recorded 644.56±20.49 and 535.56±29.49, respectively which were found to be statistically highly significant ($p<0.01$) elevation (Table 3).

Serum IL-12 level: As depicted in Table 3, infection cause an increase in the level of IL-12 (90.65±12.45) (group B) as compared to normal control group (group A) (32.41±2.19). The present study revealed that immunization by purified CP had an extraordinary significant ($p<0.05$) increasing effect on IL-12 level (50.15±9.39), while FABP immunization stimulated a moderate significant ($p<0.05$) elevation (40.54±5.21) (group E). On the other hand, immunization with CP or FABP before infection caused a significant decrease of the IL-12 level in serum (62.38±10.09 and 50.12±15.93, respectively) in comparison to infected rabbits (group B) (90.65±12.45) (Table 3).

Serum TNF-α level: There was an elevation in TNF-α level (343.26±15.93) in *F. gigantica* infected rabbits (group B) than that of normal control group

Table 3: Effect of CP and FABP immunization on serum IL-6, IL-10, IL-12 and TNF-α level in rabbits before and after infection with 25 *F. gigantica* metacercariae

Animal group	IL-6 (pg mL^{-1})	IL-10 (pg mL^{-1})	IL-12 (pg mL^{-1})	TNF-α (pg mL^{-1})
A: Normal control	13.26±2.19	82.31±8.89	32.41±2.19	284.46±15.18
B: Infected control	114.82±12.23	487.26±16.44	90.65±12.45	343.26±15.93
C: Immunized with CP	40.41±9.29**	270.71±14.21***	50.15±9.39*	290.39±17.11
D: Immunized with CP and infected	48.89±7.33ΔΔ	644.56±20.49ΔΔ	62.38±10.09Δ	300.32±21.33Δ
E: Immunized with FABP	90.33±8.24***	253.65±21.25**	40.54±5.21	240.92±22.36*
F: Immunized with FABP and infected	66.80±9.31Δ	535.56±29.49Δ	50.12±15.93Δ	323.29±33.04Δ

Significant difference $p<0.05$ (*, Δ), *Significant difference between groups A, C and E, Highly significant difference $p<0.01$(**, ΔΔ), Δ: Significant difference between groups B, D and F, Very highly significant difference $p<0.001$(***, ΔΔΔ), Data are represented as Mean±SD

(284.46±15.18). Treatment with purified E/S antigens (CP or FABP), showed no detected decrease in rabbits immunized with CP (290.17±56.11) while, there is a mild decrease at FABP immunization (group E) (240.92±22.36). Similarly, infection with CP or FABP prophylaxis recorded no change in the level of serum TNF-α in both groups (group D and F) (300.32±21.33 and 323.29±33.04, respectively) as compared to infected animals (group B) (Table 3).

DISCUSSION

Triclabendazole is the only effective drug against early stages of the parasite; however, resistance to it has been extensively reported (Villa-Mancera *et al*., 2008). Other control measures such as vaccination should be developed for sustainable control of this disease. Controlling fascioliasis by vaccination rather than chemotherapy would be a cheaper, more efficient and a reliable long term solution for the prevention of infection and eradication of its transmission. A number of molecules including cysteine proteinase (CL) CL, GST, LAP and FABP have the potency of inducing a protective response against *Fasciola* spp. in laboratory and large animal models. The enzymes belonging to the family have been studied most intensely and gave the most promising results when used as vaccine antigens (Dixit *et al*., 2008).

In the present study, *F. gigantica* E/S products (e.g., crude products, CP and FABP) were prepared and used to induce immune response in rabbits. CP was purified as a single band in 27.5 kDa by reducing SDS-PAGE. This was reasonable in comparison to those of Dixit *et al*. (2002) who reported that, serum antibodies from sheep experimentally infected with 100 *F. gigantica* metacercariae recognized 28 kDa CP as early as 2 week PI in sheep harbouring 38 flukes. The native *F. gigantica* FABP was purified from the crude extracts as described by Timanova *et al*. (1999) and subsequently used for the protection experiment as single band at 14 kDa on SDS-PAGE gel filteration under reducing condition.

Results of the present clearly demonstrate that the *F. gigantica* CP had a strong immunoprophylactic effect against *F. gigantica* in rabbits than FABP. Multiple immunizations of rabbits with CP recorded a high significant reduction in worm burden (34.7%). A reduction in worm numbers is the gold standard for anti-*F. gigantica* vaccine development as it also had an adverse effect on the liver egg count which results in lower number of eggs recovered from liver (55.5%). Our findings are in agreement with Piacenza *et al*. (1999) who observed that, immunization of sheep with native CL1 or CL2 against *F. hepatica* elicited protection levels of 33% and 34% as worm PR and of 71 and 81%, in egg output PR, respectively, while a cocktail of native CL1 and CL2 induced 60% as PR in fluke burden.

In contrast, there is no significant difference in worm burden and the mean total number of *F. gigantica* egg count observed in the immunized infected group where immunization of rabbit with FABP protected rabbits up to 23.5% reduction in worm burdens and 35.7% reduction in egg count after challenge with *F. gigantica* metacercaria It has been reported that the antigenic stimulation by the parasite FABP to the host during the course of experimental/natural infection may not be sufficient to evoke detectable antibody level in ELISA and WB, thereby suggesting that FABP as such is a weak antigen. Also, there is no significant humoral response generated against *F. gigantica* FABP in sheep, cattle and buffaloes (Raina *et al*., 2004).

E/S-derived CL proteases challenge was found to elicit a decrease in *F. hepatica* worm burden and/or maturity in sheep (Villa-Mancera *et al*., 2008). However, immunization of sheep with CL proteinase derived peptide led to a significant decrease in *F. gigantica* worm number, but failed to influence the size of the recovered worms (Jezek *et al*., 2008). On the other hand, the protective capacity of E/S could be related to its content of GST and FABP, major candidate vaccine antigens for fascioliasis (McManus and Dalton, 2006).

Additional beneficial aspects of the protection induced by the CL vaccines were observed. First, in all vaccine trials, the proportion of liver flukes that did not develop to maturity were greater in vaccinated than in non vaccinated controls. Consequently, the damage to the host's liver during acute infection was significantly reduced. Secondly, vaccination of both sheep and cattle also elicited a highly significant reduction (50-98%) of the parasite's ability to produce eggs and those eggs that were synthesized showed reduced 'hatch rates' (Mulcahy *et al*., 1998). The implications of these findings are that by reducing the parasite burden of the host and, at the same time, blocking the synthesis of viable eggs by those parasites that do survive in vaccinated animals, the vaccine would have a profound effect on pasture contamination and hence disease transmission.

The presented data obviously marked that the total IgG level increased with or without infection in groups of mice immunized with either CP or FABP in comparison to the control groups. While on the other hand IgM level was affected by infection while all other treatments are in close range. Also, IgG level was much more elevated than that of IgM. Thus, it may be concluded that IgG plays a prominent role in combating infection where as IgM was not as effective.

The comparison of the Ig isotype response between animals immunized with CP and that immunized with FABP shows a number of differences. CP immunization majorly induced a highly significant elevation to all types of Ig isotypes. Similarly, immunization with FABP was significantly elevated all types of isotypes except for IgG1 which recorded a slight increase. While, in case of immunization with either CP or FABP followed by infection, FABP was less potent in elevating Ig isotypes than CP.

Bossaert *et al.* (2000) also found that, IgG1 was significantly higher in calves challenged with single-dose-infected of *F. hepatica* to E/S products as compared to IgG2. Mulcahy *et al.* (1998) in their cattle immunization studies against *F. hepatica* found that, an elevation of IgG2 level was associated with protection. It is conceivable therefore that, the late strong IgG2 found in this study does favor cattle in elimination of the liver fluke than sheep with early and weak IgG2. However, more work needs to be done to examine the contribution of IgG2 toward protection against liver fluke.

Our results suggested that, predominance of IgG1 and IgG4 than IgG2 isotypes PI. This finding correlates with a predominance of IgG1-type Igs specific for *Fasciola* sp. antigens at different times PI which is characteristic of a TH 2 response which agrees with the findings of other investigators. Antibody responses in rats in the acute and chronic phase (1-21 week) of disease show a marked predominance of IgG1 over IgG2a isotypes. During the first week PI, IgG1 quickly increases, whereas, IgG2a slowly increases and reaches the highest values at 5-7 week PI (Girones *et al.*, 2007).

Analysis of the CL proteases-specific IgG1 antibody responses in infected cattle revealed that these antibodies do not appear in the serum until 4 or 5 week PI. Moreover, animals given a second infection 4 week after the primary infection did not exhibit any boosting of immune responses to the CL proteases. It is clear, therefore, that CLs are not highly immunogenic in the early stages of infection such that, at this stage, they may be considered 'hidden antigens'. This may be an important strategy used by the parasite to prevent antibodies being generated to critical molecules. Accordingly, vaccinating animals with CLs would elicit a high titre of anti-CL antibodies that would upset this evasion strategy and, therefore, would be detrimental to the early migratory stages of the parasite. Indeed, when animals are vaccinated with CL, a positive correlation was observed between protection and antibody titres (Mulcahy *et al.*, 1998).

It has been has previously shown that, the IgG2 response to a CL vaccine correlates with reduced worm burdens in vaccinated cattle, implying a protective role for this isotype against *F. hepatica*. These observations raise the interesting prospect that *Fasciola* parasites produce a factor (s) that can suppress IgG2 responses (Valero *et al.*, 2009).

Phiri *et al.* (2006) suggest that, these antigens may preferentially stimulate a Th2 T cell subset response. The late IgG2 response to *F. hepatica* and *F. gigantica* E/S products in cattle may indicate a delayed Th1 T cell subset stimulation (Moreau *et al.*, 1998).

The human immune response to parasitic infections exhibits different distributions of IgG subclass antibodies in different infected groups (Dunne, 1990; Maizels *et al.*, 1995). The genetic background of the infected individual and the intrinsic properties of the antigen itself and/or defined cytokines play a role in determining the main subclass of the antibody response. This study demonstrated the prominent role of specific IgG4 antibody in human fascioliasis caused by *F. gigantica*. It has been suggested that IgG4 antibodies are especially prominent in the total IgG response when antigenic exposure is chronic or associated with a Th cell type 2 responses (Garraud *et al.*, 2003; Wongkham *et al.*, 2005).

Results clearly demonstrate that immunization with CP or FABP elevate secretion of TH 1 cytokines IL-6, IL-12 and TNF-α and TH 2 cytokine IL-10 levels than the normal control group with no significant difference between CP and FABP antigens. Administration of CP or FABP induces a high significant increase in IL-6 and IL-10, while, a significant increase in IL-12 and TNF-α level in immunized groups with IL-10 predominating. In contrast, there is suppression in all cytokines level in rabbits immunized with CP or FABP and infected groups except IL-10 which was elevated than the infected control group. Thus, it could be suggested that, CP was more potent than FABP in increasing the levels of all cytokines understudy except IL-6 before infection. While on the other hand, infection post immunization with CP caused a decrease in all cytokines except for IL-10 which remained slightly but significant elevated.

The data presented in this study are supported by the idea of Molina (2005) who observed that, cattle and buffaloes infected with *F. gigantica* had a predominant Th2 response which started early in the infection. IL-6 production in these animals apparently influenced the initiation and maintenance of TH 2 immune response thereby down-regulating Th 1 response. IL-6 and IL-8 (in buffaloes) during infection with *F. gigantica* may thus be capable of exerting a cytotoxic effect against the fluke.

IL-6 and IL-8 have been shown to be involved in antibody dependent cell mediated cytotoxicity (ADCC) involving neutrophils. In fascioliasis, ADCC has been considered to be a mechanism by which flukes are destroyed, with the priming of neutrophils, macrophages,

eosinophils and mast cells by various cytokines (Hansen *et al*., 1999; Piedrafita *et al*., 2007). Therefore, cattle, buffalo and sheep, by producing IL-4, IL-6 and IL-8 during infection with *F. gigantica*, may be capable of exerting a cytotoxic effect against flukes.

In some of the infected rats, TNF-α also increased at 10 week PI which does not correspond to a typical Th2 response. TNF-α is implicated in the regulation of Th2 responses in other helminth infections, apparently regulating worm expulsion. Moreover, IL-4 and IL-10 can act synergistically, inhibiting the production of reactive nitrogen oxides which up-regulate IL-12 production and inflammatory responses (Girones *et al*., 2007).

More recently, however, we have proposed that the secreted CLs may be involved in suppression and/or modulation of Th1 immune responses and induction of non protective host Th2 responses (Dixit *et al*., 2008). An analysis of cytokine production by antigen-stimulated spleen cells of *F. hepatica* infected mice showed that these are predominantly of the Th2 type, i.e., production of IL-4, IL-5 and IL-10 but little or no IFN-γ (O'Neill *et al*., 2000). This is consistent with immunological observations in cattle which show that in the early stages of infections mixed Th1/Th2 responses are observed but as infection progresses, a Th2 response predominates (Mulcahy *et al*., 1999).

CONCLUSION

This study proved that native heterologous *F. gigantica* CP significantly protects rabbits against challenge infection with *F. gigantica*. Multiple immunizations with native *F. gigantica* CP in Freund's adjuvant resulted in high significant reduction in mean worm burden (34.7%) and liver egg count (55.5%) and elicit a high significant increase in IgM and IgG antibodies and more specifically IgG1, IgG2 and IgG4 isotypes with IgG4 isotype predominating.

These data suggest that the immunoprophylactic effect of the native *F. gigantica* CP is mediated by a mixed Th1/Th2 response. In contrast, FABP induce low significant reduction in mean worm burden (23.5%) and liver egg count (35.7%). The protection obtained with 27.5 kDa native heterologous *F. gigantica* CP was higher than that obtained with other homologous FABP candidate antigens.

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