Global Veterinaria 13 (5): 720-727, 2014
ISSN 1992-6197
© IDOSI Publications, 2014
DOI: 10.5829/idosi.gv.2014.13.05.86125

Evaluation of a Vaccine Candidate Isolated From *Fasciola gigantica* Excretory-Secretory Products in Rabbits

[1]Eman H. Abdel-Rahman, [2]Abdel-Rahman M. Bashtar,
[3]Mohey A. Hassanain, [3]Nawal A. Hassanain and [1]Nagwa I. Toaleb

[1]Department of Parasitology and Animal Diseases, National Research Center, Egypt
[2]Department of Zoology, Faculty of Science, Cairo University, Egypt
[3]Department of Zoonotic Diseases, National Research Center, Egypt

Abstract: Fasciolosis is an important trematode infection of herbivores worldwide with increasing evidence of being zoonotic disease of global importance. Vaccination studies with purified *Fasciola* antigens suggest that this approach to diminish morbidity and mortality and reduce transmission is a realistic goal. The current study was designed to study the immunogenic and immunoprophylactic properties of isolated fraction from *Fasciola gigantica* excretory-secretory products in rabbits using CNBr-Sepharose 4B affinity column chromatography. Characterization of the isolated ES fraction by SDS-polyacrylamide gel electrophoresis and isoelectric focusing showed that the fraction consists of two polypeptides of 27 and 23.5KDa compared with 9 bands associated with the crude ES antigen (14-175 KDa) and with isoelectric points of (7.2 and 6.96). The isolated fraction proved potency in the diagnosis of bovine fasciolosis using ELISA which recorded 100% sensitivity. Level of anti-ES fraction antibodies was significantly higher in rabbits vaccinated twice compared with non-vaccinated infected control. The immunoblot assay proved that the two bands associated with the isolated fraction; 27 and 23.5KDa are immunoreactive. A reduction of 85.7 % in worm burdens was recorded in vaccinated rabbits. In addition, infected control rabbits had higher gamma glutamyl transferase (GGT) levels than immunized rabbits. Lastly, gross anatomic observation showed fewer liver lesions in all vaccinated rabbits than in control. Thus, the current study introduces a successful vaccine candidate against fasciolosis which deserves further evaluation in other animal models and with different adjuvant.

Key words: *Fasciola gigantica* · Excretory-secretory products · Affinity column chromatography · Vaccine

INTRODUCTION

Fasciolosis is an infection of herbivores caused primarily by the parasitic trematodes *Fasciola hepatica* and *Fasciola gigantica*. *F. gigantica* has a worldwide distribution primarily of tropical climates in Africa and Asia. Fasciolosis is a true zoonosis with increasing evidence of human infections worldwide, but especially in the rural communities. High human infection rates have also been reported in Egypt [1] and Iran [2].

Fasciolosis in ruminants, caused by infection with the parasite *F. hepatica* (Temperate liver fluke) and *F. gigantica* (Tropical liver fluke), causing significant economic loss, estimated ≈3 billion US$ per year to rural agricultural communities and commercial producers [3].

The flukicide triclabendazole is the most effective drug for controlling fasciolosis [4]; however the cost of treatment prevents its wide use by rural producers in developing countries. Moreover, resistance to triclabendazole has been reported in sheep infected with *F. hepatica* [5]. Hence, there is a need to develop cost-effective and sustainable control strategies. This makes vaccination an important alternative in the control and morbidity reduction of fasciolosis in livestock and other herbivores. Studies in natural hosts such as sheep and cattle provide strong evidence that ruminants acquire resistance to both *F. hepatica* and *F. gigantica* infection following vaccination using irradiated metacercariae or parasite extracts [6, 7], or with defined antigens [8,9].

Corresponding Author: Nawal A. Hassanain, Zoonotic Diseases Department, National Research Center, Postal code: 12622, Egypt. E-mail: nawalhassanain@yahoo.com.

The present study aimed to purify fraction from adult *F. gigantica* excretory-secretory products. The study provides characterization and evaluation of this isolated ES fraction in diagnosis of fasciolosis by ELISA as well as evaluation of its protective potency as a vaccine candidate

MATERIALS AND METHODS

Animals: Fifty native breed rabbits (1.5-2.00 Kg) were used. Faecal samples of each rabbit were microscopically examined in the laboratory for *Fasciola* eggs [10] before the start of the experiment and they were found free from *Fasciola* and other parasitic infections.

A total number of ninety three blood samples and their corresponding faecal samples were individually obtained from buffaloes in the abattoir. Moreover, each faecal sample was microscopically examined in the laboratory for *Fasciola* eggs. Serum samples from buffaloes infected with *Fasciola* were considered as positive, while negative sera were obtained from non-infected buffaloes and all sera were stored at-20 °C until use to assess sero-diagnostic potency of isolated ES fraction.

Parasites: Adult *Fasciola* worms were collected from condemend livers naturally infected with fasciolosis from buffaloes slaughtered in Cairo abattoir.

Fasciola gigantica encysted metacercariae were purchased from Theodor Bilharz Research Institute, Egypt. The viability of the metacercariae was checked by microscopy on arrival.

Antigen Preparations: Whole adult worm (FgWWE) and Encysted metacercariae (FgEMC) extracts of *F. gigantica* were prepared according to Abdel-Rahman and Abdel-Megeed [11].

The two antigens (FgWWE) and (FgEMC) were aliquoted and stored at-20°C until use. *F. gigantica* excretory-secretory antigen (FgESA) was prepared according to method of McGonigle and Dalton [12].

Protein content of the prepared extracts was assayed according to Lowry *et al*. [13] and stored at-20°C until use.

Preparation of Rabbit Hyperimmune Serum: About 100 mg of *F. gigantica* excretory-secretory antigen (FgESA) was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into each of 5 rabbits [14]. A booster dose of FgESA in Freund's incomplete adjuvant was injected on day 14 after the first dose. Second and third booster doses were given on days 21 and 28, respectively [15]. Blood samples were collected 4 days after the last injection from rabbit's ear vein. Rabbit anti-*F. gigantica* ES antisera (RAFgESA) were aliquoted and stored at-20°C until use.

Antibody-Sepharose 4B Affinity Chromatography: The prepared hyperimmune serum (RAFgESA) was dialyzed and coupled to the cyanogen bromide (CNBr)-activated Sepharose-4B at the ratio of 2mg/ml-swollen beads by strictly following the manufacturer instructions.

Affinity Purification of Adult *F. gigantica* ES Antigen: Crude ES (FgESA) was applied to the column composed of CNBr-Sepharose 4B coupled with RAFgESA and allowed to mix overnight at 4°C in a rotary mixture. The column was washed with 0.15 M PBS pH 7.3 several times, till the unbound part of the antigen has passed completely from the gel. The bound material was eluted with 50 mM glycine-500 mM Nacl-0.02 % w/vNaN_3pH 2.3. The eluted fraction was immediately brought to pH 8.0 with solid $NaHCO_3$ and then dialyzed against 0.03 M PBS-0.02% w/v NaN_3 pH 8.0. The isolated ES fraction was assayed for protein content by the method of Lowery *et al*. [13].

Antigen Characterization

SDS-PAGE: The four antigens; FgWWE, FgEMC, crude FgESA and isolated ES fraction were separately electrophoresed on SDS-PAGE slab gel according to procedures of Laemmli [16]. After separation, slab gel was stained with silver stain according to Wray *et al.* [17]. High and low molecular weight standards (RPN756-Amersham Pharmacia biotech) were electrophoresed on the same gel to calculate the relative molecular weights of the examined antigens. Gel was photographed wet using Kodak Tri-X-pan films.

Isoelectric Focusing (IEF): IEF of *F. gigantica* isolated ES fraction was performed as described by O'Farrell [18] in slab gel supplemented with urea and ampholine. Gel was stained with Coomassie blue and photographed wet. Isoelectric points (PIs) of the bands of *F. gigantica* isolated ES fraction were determined by running a mixture of proteins of known isoelectric points on the same gel (IEF mix 4.6-9.3).

Immunization and *F. gigantica* Infection Protocols in Rabbits: Rabbits were divided into two groups, the first group consisted of thirty rabbits (Vaccinated infected

group), while, the twenty rabbits in the second group were non-vaccinated infected (As control). The first group was injected subcutaneously with 40µg, for each rabbit, of the isolated ES fraction (As vaccine) emulsified in complete Freund's adjuvant for primary immunization [19]. This immunization was boostered two weeks later with another 40µg of the same protein in incomplete Freund's adjuvant. Each rabbit was infected orally with 30 *F. gigantica* metacercariae, in the vaccinated and non-vaccinated groups (Groups 1and 2) after two weeks of the second immunization [19]. Rabbits were bled prior to immunization and at 2 week intervals after immunization until the end of the study for the collection of sera. All rabbits were necropsied at 10 weeks Post infection for the determination of worm burdens and their sizes.

Evaluation of Diagnostic Potency of *F. gigantica* Fraction by ELISA: ELISA plates were coated with isolated ES fraction using carbonate buffer pH 9.6 according to procedures of Santiago and Hillyer [20], with some modifications. Antigen concentration, bovine serum samples and anti-bovine IgG horse radish peroxidase dilutions were determined by checkerboard titration. The reaction was read using ELISA-reader at 405 nm.

Evaluation of Protective Potency of *Fasciola* Vaccine Candidate: The rabbits were bled prior to immunization and at 2 weeks intervals after immunization until the end of the study for the collection of sera. The humoral response elicited by the immunization and infection was analyzed by ELISA where ELISA plates were coated with FgWWE, FgEMC, crude FgESA and isolated ES fraction separately and serum samples from vaccinated infected rabbits and from non-vaccinated infected ones were investigated. Anti-rabbit IgG horseradish peroxidase was used and reactions were read by ELISA reader at 405nm.

Immunobloting Assay: After electrophoresis, FgWWE, FgEMC, crude FgESA and isolated ES fraction were blotted onto nitrocellulose membrane according to Towbin *et al.* [21] in a blotting system. The nitrocellulose membrane was incubated with sera collected after two weeks post vaccination from rabbits vaccinated with isolated ES fraction and sera collected at ten weeks post challenge of rabbits with encysted metacercariae (Diluted at 1:50). The membrane was then incubated with anti-rabbit IgG horse-radish peroxidase conjugate at a dilution of 1:3000. ECL Western blotting reagents (Amersham, UK) were utilized to visualize the immunoreactive bands.

Evaluation of Hepatic Damage: At necropsy hepatic damage was evaluated subjectively by the observation of macroscopic alterations of the organ including the following: color change to grayish white, increase in size and consistency, dilation and increased thickness of the bile ducts, the presence of mucopurulent deposits and the formation of surface scars. The grade of lesions observed was summarized semiquantitively using four stages symbolized by 1 through 4, plus symbols that expressed the intensity and extension of the alterations observed as following: + mild; ++ moderate; +++intense and ++++severe. The grading was carried out by an experienced pathologist who performed the scoring without knowing to which group each rabbit belonged.

As another measure of hepatic damage, the activities in sera of two cytolysis enzymes GOT (L-Aspartate 2 exoglutarate aminotransferase, [AST]) and GPT (L-Alanine 2 oxoglutarate aminotransferase, [ALT]) and one cholestasis enzyme GGT (Gamma glutamyl transpeptidase) were determined using a Hitachi 912 auto analyzer.

RESULTS

Purification of Species Specific Antigen from FgESA: The purification of FgESA was attempted by affinity chromatography using CNBR-Sepharose 4B column coupled with RAFgESA producing one fraction.

Structural Characterization of the Isolated ES Fraction: The electrophoretic profile of isolated ES fraction in comparison with the FgWWE, FgEMC and FgESA was shown in Fig. 1 A, B, C and D respectively. The isolated ES fraction was resolved under reducing conditions into only two bands with molecular weight of 27 and 23.5KDa (Fig. 1, lane D). And the isolated ES fraction had pIs 7.2 and 6.96 as shown in Fig. (2), Lane A.

Immunoblot Assay: The two bands of the isolated fraction reacted positively with rabbit serum samples collected at two weeks post immunization and ten weeks post challenge (Fig. 3 and 4 lane D) while different profiles were detected with the other antigens based on the reacted serum (Fig. 3 and 4 lanes A, B and C) respectively.

Assessment of Protection

Post Mortem Examination of All Animals: Statistically significant results regarding fluke burden in the two rabbit groups were observed revealing 85.7% reduction in worm burden which detected in eleven vaccinated infected

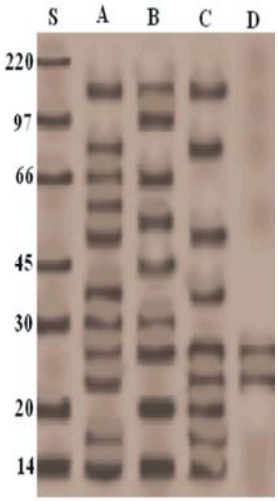

Fig. 1: Electrophortic profile of: FgWWE (Lane A), Fg EMC (Lane B), FgESA (Lane C), isolate ES fraction (Lane D) and Molecular weight standards (lane S)

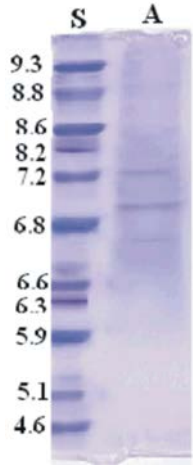

Fig. 2: Isoelectric focusing of isolated ES fraction (Lane A). Isoelectric focusing standards (Lane S)

rabbits, where the worms recovered were ranged 1-3 worm / animal. In the other 19 vaccinated infected rabbits no flukes were detected, which consequently, recorded 63.3% complete protection. Concerning worms recovered from non-vaccinated infected rabbits (Control) 13-15 worms / animal were collected.

Liver Lesions: Evaluation of macroscopic liver lesions revealed that the rabbits vaccinated with pure fraction had fewer mild liver lesions and the livers looked normal and healthy than the non-vaccinated infected rabbits. While, the non-vaccinated infected rabbits revealed alterations in the liver including change of the color to grayish white, increase in size and increase the thickness of bile ducts.

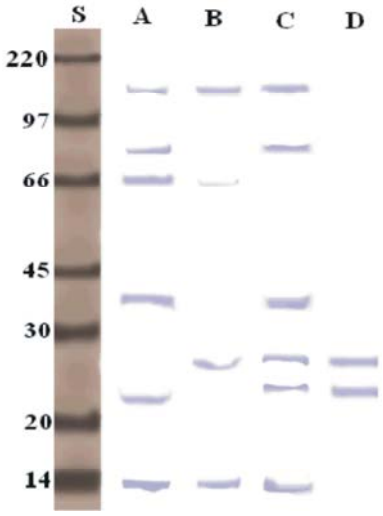

Fig. 3: Immunoreactive bands identified by sera from rabbits at two weeks post vaccination using immunoblot assay: FgWWE Lane A, FgEMC Lane B, FgESA Lane C and isolated ES fraction Lane D. Molecular weight standards in KDa Lane S.

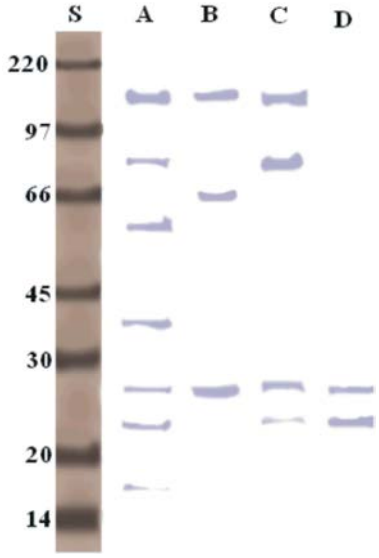

Fig. 4: Immunoreactive bands identified by sera from vaccinated infected rabbits at ten weeks post challenge using immunoblot assay: FgWWE Lane A, FgEMC Lane B, FgESA Lane C and isolated ES fraction Lane D. Molecular weight standards in KDa Lane S.

IgG Response: The level of IgG response of the vaccinated infected rabbits to the isolated ES fraction was higher than the response toward FgWWE, FgEMC and Crude ES (FgESA) at all intervals before and after challenge (Fig. 5).

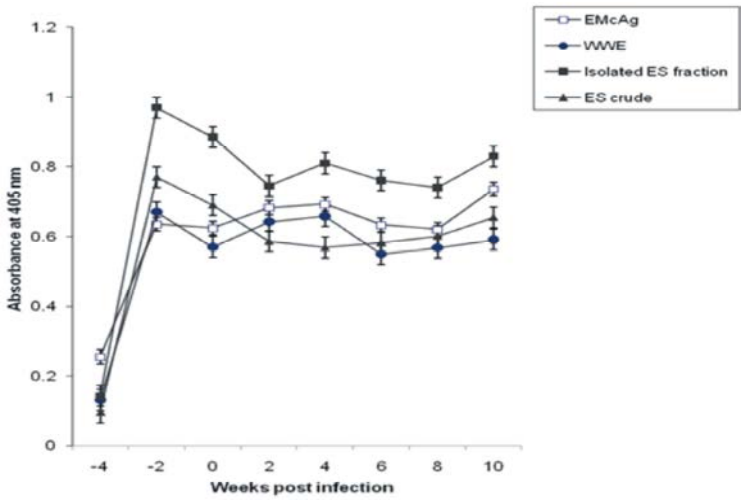

Fig. 5: levels of IgG antibody response measured by ELISA in immunized rabbits. *F. gigantica* crude whole worm extract (● FgWWE), *F. gigantica* crude encysted metacercariae extract (□ FgEMC), Crude excretory-secretory (▲ FgESA) and isolated ES fraction (■ Pure fraction). Standard error bars are shown.

Liver Function: Significant differences between immunized and control infected rabbits were observed in the levels of GGT at 8 weeks after infection. At this time, the levels of GGT were higher in non-immunized rabbits than in immunized ones. However, slight differences were observed in the levels of GOT and GPT between the immunized and non-immunized groups.

Potency of Isolated ES Fraction in Diagnosis of Fasciolosis: ELISA was adopted to evaluate the potency of the isolated ES fraction in the diagnosis of fasciolosis among buffaloes. All the positive buffalo serum samples reacted positively with the specific bound fraction (100% sensitivity). OD values of the examined serum samples ranged from (0.050 to 0.764).

DISCUSSION

In the current research the evaluation and characterization of the prepared products FgESA was done by SDS-PAGE which showed a number of bands with different molecular weights of 175, 80, 51, 36.4, 27, 23.5, 20, 16.7 and 14 KDa. This profile indicates the complex structure of this trematode species and agrees with Silva *et al*. [22] and Arafa *et al*. [23].

In the current study, a purification of ES products of *F. gigantica* adult fluke was performed by CNBr-sepharose 4B affinity column chromatography using antibodies to parasite which offers a good opportunity for isolation of specific antigenic determinants. The present investigation revealed the isolation and characterization of ES fraction as identified by SDS-PAGE which showed two bands with molecular weight 27 and 23.5 KDa. Whereas, the purification of *Fasciola* ES products was previously probed by conventional gel filtration and HPLC [24] which in contrary to the current results, indicated many fractions with different molecular weights; 150-160 KDa; 25-48 KDa and 12-14 KDa using SDS-PAGE. Comparable approach was adopted by Estuningsih *et al*. [25] who purified 4 native antigens either from whole *F. gigantica* extract or its products using affinity column chromatography. The FgGST migrates at 28-30 KDa in SDS-PAGE gels; FgCat L migrates as a doublet at 27-28 KDa; FABP is a complex of proteins migrate at 14 KDa and recombinant FABP consists of a single major band migrated at 94 KDa.

The isolated ES fraction in the present work was also characterized according to its isoelectric points by isoelectric focusing. Interestingly, the assay revealed also two main bands of approximately PIs 7.2 and 6.96. This observation supports the postulation that the fraction consists of only two bands.

The current ES fraction showed 100% sensitivity in the diagnosis of bovine fasciolosis. This observation proved the success of the purification process in isolating the most immunogenic fraction of ES products which is supported with the sensitivity of ELISA. Comparable results were recorded in the diagnosis of bovine fasciolosis using glycoprotein fraction of *F.gigantica* mature worms in ELISA [26]. The glycoprotein fraction

recorded 95% sensitivity and the authors attributed this high sensitivity to the efficacy of the fraction and sensitivity of ELISA. In the present work, a high level of IgG was detected in the sera of rabbits subcutaneously vaccinated with the isolated ES fraction. This observation reflected the potency of this fraction to elicit a strong primary response. Immunization with this fraction followed by challenge with *F. gigantica* metacercariae resulted in high level of IgG to ES fraction at two weeks post first immunization. Then the level of IgG was gradually decreased until two weeks post infection and then increased again at four weeks post infection and remained stable to the end of the experiment, although it was higher than that in non-vaccinated rabbits; and higher than that to other used antigens (FgWWE, FgEMC, FgESA).This observation explained the reason behind the recorded protection. In previous study, Abdel-Rahman and Abdel Megeed [27], introduced successful vaccine candidate against fasciolosis recording 66.6% protection in rabbits and was isolated from coproantigen of *F. gigantica* by ion exchange chromatography. The current candidate resulted in higher protection than the coproantigen fraction; 85.7% which is probably attributed to the different sources of the antigen and the purification approaches.

Two immunogenic bands of 27 and 23.5 KDa were identified in the isolated fraction with both sera collected early after immunization and late at the end of the experiment. These two bands were probably responsible for the highest diagnostic potency and protection value of the fraction. Attallah *et al*. [28] indicated that two highly immunoreactive bands; 26 KDa and 28 KDa were identified not only in an extract of adult *F. gigantica* but also in the ES products of the worms. Furthermore, Tantrawatpan *et al*. [29] has been used partially purified 27 KDa ES antigen from *F. gigantica* adult worm (Fg27) as the sensitive and specific antigen for immunodiagnosis of human fasciolosis.

In the current research SGOT remained elevated, while SGPT declined during the course of the infection. This observation agreed with Timoteo *et al*. [30]. SGOT is characteristic of the liver damage caused by the parasite in alpacas and rabbits as previously observed in cattle [31]. In addition, Nambi *et al*. [32] found that serum AST (SGOT) is an indicator of liver cell damage, at 30 day post infection in the infected control group than vaccinated calves, but declined in the subsequent weeks PI. This correlates with the migratory stages of the flukes when maximum damage to the liver tissue occurs. However, AST level in vaccinated calves was slightly different than negative control animals, suggesting that immunization prevented liver damage by flukes. Moreover, a drastic change in all the measured parameters in mice from which liver function enzymes such as AST (AGOT), ALT (SGPT) and alkaline phosphatase (ALP), after *S. mansoni* infection and a noticeable improved level after vaccination with FhES antigen was observed [33].

The facts that important potential vaccine candidates against fasciolosis have been identified suggested that now the time is ripe to look at combinations of these successful candidates as chimeric proteins or as DNA prime and peptides boost vaccines. Recombinant homologues can be identified and obtained by numerous expression systems that are widely available [34]. The future of fasciolosis vaccines in farm animals looks bright and research in this field should be encouraged. But the major difficulty for studies on vaccines for this orphan disease still lack funding.

REFERENCES

1. Curtale, F., E.S. Hammoud, A. Wakeel, S. Mas-Coma and L. Savioli, 2000. Human fasciolosis, an emerging public health problem in the Nile delta, Egypt. Res. Rev. Parasitol., 60: 129-134.
2. Moghaddam, A.S., J. Massoud, M. Mahmoodi, A.H. Mahvi, M.V. Periago, P. Artigas, M.V. Fuentes, M.D. Bargues and S. Mas-Coma, 2004. Human and animal fasciolosis in Mazandaran province, northern Iran. Parasitol. Res., 94: 61-69.
3. Espino, A.M. and B.E. Duménigo, 2003. Fasciola hepatica: Immunodiagnosis, Pathology and Control. International Handbook of Foodborne Pathogens, Marcel Dekker, Inc. Chapter, 32: 539-562.
4. Suhardona, S. Widjajanti, P. Stevenson and I.H. Carmichael, 1991. Control of Fasciola gigantica with triclabendazole in Indonesian cattle. Trop. Anim. Health. Prod., 23: 217-220.
5. Overend, D.J. and F.L. Bowen, 1995. Resistance of Fasciola hepatica to triclabendazole. Aust. Vet. J., 72: 275-276.
6. Spithill, T.W., P.M. Smooker, J.L. Sexton, E. Bozas, C.S. Nirrusib, J. Creany and J.C. Parsons, 1999. Development of vaccines against Fasciola hepatica. In: Fasciolosis (ed. J.P. Dalton,) Wallingford, oxon, CAB International, pp: 377-410.

7. Abán, J.L., P. Casanueva, J. Nogal, M. Arias, P. Morrondo, P. Diez-Baños, G.V. Hillyer, A.R. Martinez-Fernández and A. Muro, 2007. Progress in the development of Fasciola hepatica vaccine using recombinant fatty acid binding protein with the adjuvant adaptation system ADAD. Vet. Parasitol., 145: 287-296.
8. Chantree, P.1., M. Phatsara, K. Meemon, P. Chaichanasak, N. Changklungmoa, P. Kueakhai, N. Lorsuwannarat, K. Sangpairoj, S. Songkoomkrong, C. Wanichanon, T. Itagaki and P. Sobhon, 2013. Vaccine potential of recombinant cathepsin B against Fasciola gigantica. Exp Parasitol., 135(1): 102-109.
9. Villa-Mancera, A.1., A. Reynoso-Palomar, F. Utrera-Quintana and L. Carreón-Luna, 2014. Cathepsin L1 mimotopes with adjuvant Quil A induces a Th1/Th2 immune response and confers significant protection against Fasciola hepatica infection in goats. Exp Parasitol., 135(1): 102-109.
10. Thienpont, D., F. Rochete and O.F. Vanprijs, 1979. Diagnosing helminthiasis through coprological examination. Janssen Res. Found., Beerse, Belgium, pp: 180.
11. Abdel-Rahman, E.H. and K.N. Abdel-Megeed, 2000. Molecular identity of major cross-reactive adult antigens in Fasciola gigantica; Toxocara vitulorum and Moniezia expansa. J. Egypt. Soc. Parasitol., 30: 561-571.
12. McGonigle, S. and J.P. Dalton, 1995. Isolation of *Fasciola hepatica* haemoglobin. Parasitol., 111: 209-215.
13. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.
14. Guobadia, E.E. and B.O. Fagbemi, 1997. The isolation of Fasciola gigantica-specific antigens and their use in the serodiagnosis of fascioliasis in sheep by the detection of circulating antigens. Vet. Parasitol., 68: 269-282.
15. Fagbemi, B.O., I.O. Obarisiagbon and J.V. Mbuh, 1995. Detection of circulating antigen in sera of Fasciola gigantica-infected cattle with antibodies reactive with a Fasciola-specific 88 KDa antigen. Vet. Parasitol., 58: 235-246.
16. Laemmli, U.K., 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature, 227: 680-685.
17. Wray, W., T. Boulikas, V.P. Wray and R. Hancock, 1981. Silver staining of proteins in polyacrylamide gel. Anal. Biochem., 118: 197-203.
18. O'Farrel, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., 250: 4007-4021.
19. Muro, A., V. Ramajo, J. Lopez, F. Simon and G.V. Hillyer, 1997. Fasciola hepatica Vaccination of rabbits with native and recombinant antigens related to fatty acid binding proteins. Vet. Parasitol., 69: 219-229.
20. Santiago, N. and G.V. Hillyer, 1988. Antibody profiles by EITB and ELISA of cattle and sheep infected with Fasciola hepatica. J. Parasitol., 74: 810-818.
21. Towbin, H., T. Stachelin and J. Gordon, 1979. Electrophoretic transfer of proteins from polacrylamide gels to nitrocellulose sheets: Procedure and some application. Proc. Nat. Acad. Sci. USA, 179: 4350-4354.
22. Silva, M.L.S., J.M.C. Dacosta, A.M.V. Dacosta, A.M. Viana, M.A. Pires and S.A. Lopes, 1996. Antigenic components of excretory-secretory products of adult Fasciola hepatica recognized in human infections. Am. J. Trop. Med. Hyg., 45: 146-148.
23. Arafa, M.S., S.M. Abaza, K.A. EL-Shewy, E.W. Mohareb and A.A. EL-Moamly, 1999. Detection of Fasciola-specific excretory/ secretory (ES) protein fraction band (49.5 KDa) and its utilization in diagnosis of early fascioliasis using different diagnostic techniques. Egypt. J. Soc. Parasitol., 29: 911-926.
24. Rivera Marrero, C.A., N. Santiago and G.V. Hillyer, 1988. Evaluation of immunodiagnostic antigens in the excretory-secretory products of Fasciola hepatica. J. Parasitol., 74: 646-652.
25. Estuningsih, S.E., P.M. Smoker, E. Wiedosari, S. Widjajant, S. Vaiano, S. Partoutomo and T.W. Spithill, 1997. Evaluation of antigens of Fasciola gigantica as vaccines against tropical fasciolosis in cattle. Int. J. Parasitol., 27: 1419-1428.
26. Eman, H. Abdel-Rahman, Azza H. Mohamed, Adel A.H. Abdel-Rahman and Eman E. El Shanawany, 2014. The role of Ser-(Arg-Ser-Arg-Ser-GlucNAc)19-GlucNAc Fasciola gigantica glycoprotein in the diagnosis of prepatent fasciolosis in rabbits. J. Parasit. Dis. (In Press).
27. Abdel-Rahman, E.H. and K.N. Abdel-Megeed, 2004. Fasciola gigantica: Immunization of rabbits with proteins isolated from coproantigen. J. Egypt. Soc. Parasitol., 34: 631-642.

28. Attalah, A.M., E.A. Karawia, H. Esmail, A.A. Tabll, A.A. Nawar, W.A. Ragab, M.M. Abdel Aziz and I. El-Dosky, 2002. Identification and characterization of a 26 to 28 KDa circulating antigen of Fasciola gigantica. Ann. Trop. Med. Parasitol., 96: 271-282.
29. Tantrawatpan, C., W. Maleewong, C. Wongkham, S. Wongkham, P.M. Intapan and K. Nakashima, 2003. Characterisation of Fasciola gigantica adult 27-KDa excretory-secretory antigens in human fasciolosis. Parasitol. Res., 91: 325-327.
30. Timoteo, O., V. Maco, J.R.V. Maco, V. Neyra, P.J. Yi, G. Leguia and J.R. Espinoza, 2005. Characterization of the humoral immune response in alpacas (Lama pacos) experimentally infected with Fasciola hepatica against cysteine proteinases FaS1 and FaS2 and histopathological findings. Vet. Immunol. Immunopathol., 105: 77-86.
31. Leclipteux, T.H., P.R. Torgerson, M.L. Doherty, D. McCole, M. Protz, F. Farnir and B. Losson, 1998. Use of excretory-secretory antigens in a competition test to follow the kinetics of infection by Fasciola hepatica in cattle. Vet. Parasitol., 77: 103-114.
32. Nambi, P.A., S.C. Yadav, O.K. Raina, D. Sriveny and M. Saini, 2005. Vaccination of buffaloes with *Fasciola gigantica* recombinant fatty acid binding protein. Parasitol. Res., 97: 129-135.
33. Hamed, M.A., 2006. Excretory-secretory product of Fasciola hepatica worm protects against Schistoma mansoni infection in mice. Indian J. Exp. Boil., 44: 554-561.
34. Dalton, J.P., P.J. Brindley, D.P. Knox, C.P. Brady, P.J. Hotez, S. Donnelly, S.M. O'Neill, G. Mulcahy and A. Loukas, 2003. Helminth Vaccines: from mining genomic information for vaccine targets to systems used for protein expression. Int. J. Parasitol., 33: 621-640.