

## Preparation of Three *Fasciola gigantica* antigens and determination of their efficacy as a vaccine for protection against fascioliasis in Rabbits

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### الخلاصة

تضمنت الدراسة الحالية تحضير لقاح لداء الوريقات الكبدية من ثلاثة أنواع من المستضدات لطفيلي *Fasciola gigantica* هي المستضد الابرزي-الافرازي excretory-secretory antigen (ESA) والمستضد الجسمي (SA) somatic antigen و إنزيم الكاثبسين المنقى جزئياً partially purified cathepsin (P.P.CatA) ، وإجراء اختبار فعالية اللقاح في المضائف التجريبية (الأرانب)، إذ استخدم (12) أرنباً محلياً بعمر شهرين وبأوزان متشابهة، قُسمت إلى ثلاث مجاميع (كل مجموعة تضم أربعة حيوانات) وكل مجموعة لقحت بإحدى المستضدات المستحلبة بالعوامل المساعدة Freund adjuvant ومقارنتها بمجاميع السيطرة والتي تضم 4 أرانب لقحت بالـ PBS و 4 أخرى تركت بدون تلقيح، وكما شملت الدراسة إصابة الحيوانات الملقحة بالمستضدات والسيطرة (الملقحة بـ PBS) بـ 90 مذنبه متكيسة والتي تم الحصول عليها مختبرياً من إصابة المضيف المتوسط (*Lymnaea auricular*) وقد شرحت الحيوانات بعد مرور 12 أسبوع من الإصابة. تم قياس تركيز البروتين للمستضدات المحضرة والوزن الجزيئي لمستضد P.P.Cat، إذ بلغ 1.5 ملغم بروتين/ملتر لمستضد SA و 1.2 ملغم/ملتر لمستضد ESA و 0.04 ملغم/ملتر و 27.6 كيلو دالتون لمستضد P.P.Cat. وحددت الحماية بإتباع عدد من الاختبارات لمعرفة الفروق بين المستضدات قيد الدراسة وكان أولها تقدير تحطيم الكبد مظهرياً وبالاعتماد على فهرس حز تحطيم الكبد، وقد اتضح من النتائج بان هناك فروقاً معنوية وبمستوى أهمية ( $P < 0.05$ ) في جميع المجاميع الملقحة بالمقارنة مع السيطرة. تم حساب أعداد المخمرات وقدرت نسبة الاختزال، إذ ازدادت نسب الاختزال مع نقصان عدد المخمرات وبلغت أعلى قيمة لها 81.3% باستخدام P.P.CatA في حين بلغت أقل نسبة للاختزال 39.53% باستخدام SA وهذا بمثابة الاختبار الثاني الذي اتخذ لمعرفة الفروق بين المستضدات، كما أظهرت نتائج الاختبار الثالث المتبع، أي الفروق المظهرية في عب الديدان Fluke burden (طول ووزن) بان هناك فروقاً معنوية في أطوال الديدان للحيوانات الملقحة بـ P.P.CatA مقارنة مع السيطرة أما ببقية القياسات وعدد البيوض فلم تظهر فروقاً معنوية، كما تم التحري عن نسب الاختزال وحيوية البيوض وأظهرت النتائج وجود فروق معنوية وبمستوى أهمية ( $P < 0.01$ ). استخدم اختبار الامتصاصية المناعية المرتبطة بالإنزيم ELISA لتحديد مستوى الضد IgG في مصول الحيوانات الملقحة والسيطرة، وقد لوحظ ارتفاع في مستوى الضد بعد أسبوع من الحقنة الأولى والذي ازداد بعد الحقنة الثانية والثالثة ثم بدا بالتناقص في يوم الإصابة والذي ازداد تدريجياً بعد أسبوعين من الإصابة ثم تناقص في الأسبوع السادس وبعد ذلك بقي مستواه ثابتاً حتى نهاية التجربة، مقارنة مع مصول السيطرة التي لم يرتفع مستوى IgG فيها قبل الإصابة ولوحظ ارتفاعه في الأسبوع الثاني ثم تناقص بعد الأسبوع الثامن وبعد ذلك بقي ثابتاً حتى يوم التشريح. وبصورة عامة يمكن القول ان هذه الاختبارات قد أظهرت عدداً من الفروقات التي يمكن اعتمادها كأدلة على أفضلية P.P.CatA كلقاح يليه ESA ثم SA.

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## Abstract

This study was included the preparation of vaccine against fascioliasis from three *Fasciola gigantica* antigens, somatic antigen, excretory-secretory antigen and partially purified cathepsin antigen. Their vaccine efficacy was tested in rabbits, by using 12 *Lepus lepus arabica* rabbit, aged 2 months, similar weight.

The rabbits were subdivided to three groups each of four rabbits and was vaccinated with one antigen emulsified in Freund adjuvant. In compare to control groups Which involved 4 rabbits vaccinated with PBS and other four left without vaccination, challenge infection was done by vaccination the animals with 90 metacercaria obtained from experimentally infected intermediate host. All animals were killed 12 weeks after challenge. Protein concentration of all prepared antigens and the molecular weight of P.P.CatA was also measured, which reached (1.5, 1.2 and 0.04) mg/ml in SA, ESA and P.P.CatA respectively, while P.P.CatA 26.7 KD.

Protection estimated by followed number of aspects, the first aspect of this study, was the determination the hepatic damage which was evaluated using gross morphometric observations depending upon the severity and intensity of lesions. Significant differences ( $p < 0.05$ ) was noted between vaccinated and control groups.

The second aspect was concerned with estimation of fluke number and reduction rate, the high rate (81.3%) recorded in animals vaccinated with P.P.CatA while (39.53%) of those vaccinated with SA. Significant differences ( $p < 0.05$ ) was documented in the length of fluke in animals vaccinated with P.P.CatA. No significant differences were noted in fluke burden (width, length, weight) and fecal egg counts, Significant differences ( $p < 0.01$ ) were noted in egg viability and reduction rate in the vaccinated animals in comparison with control group.

ELISA was used for monitoring of IgG levels in all vaccinated and control animals, before and after infection. The IgG increased in the sera of animals vaccinated with SA, ESA and P.P.CatA within 1 week after the first injection. A boosting of the antibody responses was observed in all groups following both the second and third immunization.

An increasing in the antibody titers in all vaccinated animals was observed within 2 weeks following challenge. IgG in all vaccinated groups remained high throughout the infection but decreased at approximately 6 weeks after infection, and then remained constant throughout the subsequent weeks. Antibodies to antigens in the sera of the control group increased within the

first 2 to 4 weeks after infection but decreased at approximately 8 weeks and then remained constant throughout the subsequent weeks.

Accordingly, the results of these aspects could be consider a P.P.CatA as a first best vaccine then secondly ESA.

## Introduction

The liver flukes, *Fasciola hepatica* and *Fasciola gigantica*, cause infectious disease predominately in ruminants, causes significant economic loss estimated at US\$ 2000M per annum to rural agricultural communities and the commercial sector worldwide (1). Fasciolosis is increasingly recognized as causing significant human disease, with 2.4 million people infected (2,3).

Humans are often infected in communities where there is close human-ruminant interaction, such as in some South American communities, Egypt, Iran and Iraq (4). The geographical range of these two parasite species (temperate and tropical, respectively) ensures worldwide infections. Infection is primarily acquired by the ingestion of vegetation on which metacercariae are encysted. Within the duodenum the metacercariae excysted, penetrate the intestinal wall, and then migrate via the peritoneal cavity to the liver. Here, the immature flukes spend 7 to 12 weeks migrating through the tissue causing extensive hemorrhage and fibrosis before they move into the bile ducts and mature to adults. Mature flukes produce numerous egg which are deposited on pastures in the feces. Miracidia hatch from the eggs and penetrate an intermediate snail host, from which the infective metacercariae erupt after a developmental period(5).

Although anthelmintic treatment is effective against disease, this is an expensive and nonsustainable measure, and drug resistant strains have been reported (6,7). The alternative to anthelmintics is the development of a vaccine. Vaccines represent the most attractive long-term alternative to solve this dilemma, as they represent an environmentally-friendly method for the control of liver fluke disease in live stock (8). However, despite several decades of research, there is no commercial vaccine available for fasciolosis(9).

There have been many attempts to vaccinate animals with various liver fluke extracts, such as crude somatic antigens and excretory/secretory antigens, but also with irradiated attenuated vaccines and various defined antigens (10). The mean level of reduction in worm burdens observed in cattle immunized with different antigens was range of 43-72%, suggesting that the control of fasciolosis by immunological intervention may be an achievable

goal (10). Recently, the search for the development of an effective vaccine against *Fasciola* has focused on essential enzymes.

Cysteine proteases are important facilitators of virulence in flukes (11). They are produced by all stages of the fluke life cycle and may facilitate biological functions such as excystments, tissue invasion and immune evasion (12). Multivalent vaccination against fasciolosis has been attempted. Dalton *et al.*(1996) showed that combination vaccines (catL2 plus hemoglobin) gave higher efficacy in cattle than either antigen alone, however, in sheep a combination of leucine aminopeptidase (LAP) and catL vaccines were not more efficacious than LAP alone (13). However, a preliminary trial in cattle, using native cathepsin, was not successful in inducing protection against fluke burden but attributed to the production of higher titres of anti-cat antibodies (14).

The present work had been performed to study the protective value of *F.gigantica* somatic, excretory/secretory and partially purified cathepsin antigens against fasciolosis in rabbits as a highly susceptible host to *F.gigantica*.

### **Materials and methodes**

**Animals:** Sixth rabbits, 2 months old, each weighing approximately 2 Kg were used in this study, obtained from animal house of biology department, Basrah, Iraq. They were routinely examined for intestinal helminthes and Ectoparasites.

### **Preparation of antigens**

***F.gigantica* somatic antigen:** *F.gigantica* somatic antigen was prepared according to (15) from the live adult *F.gigantica* worms collected from fresh condemned buffaloes' livers at Basrah abattoir.

***F.gigantica* excretory-secretory antigen (ESA):** *F.gigantica* ES antigen was prepared from living flukes collected from fresh condemned buffaloes' livers at Basrah abattoir according (16).

***F.gigantica* P.P.Cat antigen:** Cathepsin was purified to homogeneity from the excretion-secretion (ES) products of mature flukes as previously described (16). Briefly, mature flukes were washed six times in 0.1M phosphate buffer saline (PBS), pH7.3, containing 2% glucose, 30 Mm HEPS and 25 mg gentamycin per ml. The flukes were removed, and the culture medium was centrifuged at 15000 Xg for 1 h at 4<sup>0</sup>C. The supernatant (containing ES products) was then collected, filtered and stored at -20<sup>0</sup>C until used. ESA, dialyzed, were applied to a Sephadex G-100 column. Fraction of 3 ml were collected and assayed for cathepsin activity, using gelatin substrate, fractions

containing enzyme activity were pooled and dialyzed against distilled water freeze –dried and stored at -20<sup>0</sup>C. For molecular determination, the *F.gigantica* PPCat was analyzed under reducing condition 12% SDS-PAGE with lanes loaded with 100 ug protein and subsequently stained with coomassie blue (17).

Protein concentration were determined by Lowery protein (18) assay for all antigens preparation.

**Formulation, Preparation and administer of vaccines:** The vaccine prepared according to (11). On the day before vaccine administration the powder was reconstituted in PBS and the vaccines were formulated by mixing 1 ml of the reconstituted antigen with an equal volume of freund's complete adjuvant (FCA) or freund's incomplete adjuvant (FIA). The mixture was emulsified by sonication.

**Vaccination of rabbits:** The rabbits were divided into four groups, each with four animals. Group1 animals received 150 µg of somatic antigen per immunization, group2 animals received 100 µg of excretory-secretory antigen per immunization,

group 3 animals received 50 ug of partially purified cathepsin per immunization and group 4 control animals received 1 ml PBS per immunization. Each animal received first injection of 1 ml of vaccine preparation formulated in FCA subcutaneously in day 0. A second injection on day 14 (2 weeks later) and third injection on day 21 (3 weeks later) both formulated in FIA, blood sample were taken prior to vaccination and every week through vaccination and every two week during infection.

**Parasite challenge:** Metacercariae used in this study were obtained in our laboratory by passage through the intermediate host *Lymnaea auricularia* and maintained on water and stored at 4<sup>0</sup>C. Rabbits were challenged orally at two week post third injection with 90 metacercariae (less than 3 months old) in 1 ml water using a gavage needle.

**Assessment of protection:** Twelve weeks after the oral infection, rabbits were killed and their livers removed. Pathological lesions of the liver were observed by the naked eye and a damage score assigned. The degree of damage was scored 0-5 depending upon the severity and the intensity of lesion (19). Following scoring, live adult liver flukes inside the lumen of the bile duct were removed and the whole liver sectioned into small pieces, submerged in water at 37<sup>0</sup>C for 1 h and filtered through a 200 UM mesh sieve for detection and counting of liver flukes in the liver parenchyma.

All fluke were enumerated weighed and measured the body length from head to tail and body width of each fluke

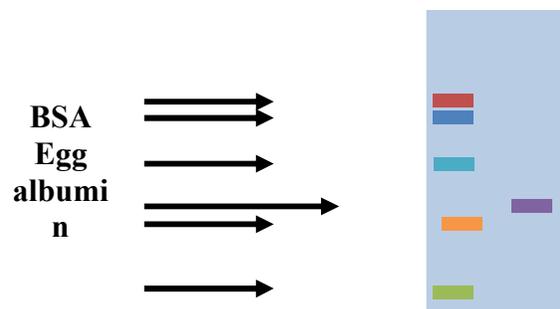
**Faecal egg and egg viability:** Faecal egg counts were carried out using the sedimentation technique as described previously (20) and egg viability was estimated accordance to the procedure described by (11).

**ELISA analysis:** ELISA plates were coated with SA, ESA and PPCatA antigen at 5,7,3  $\mu\text{g/ml}$  in carbonate binding buffer Ph9.6 and incubated overnight at 4<sup>0</sup>C. After washing with PBS/Tween20 (0.05%), plates were blocked with 2% bovine serum albumin in PBS/Tween20 for 1 h at 37<sup>0</sup>C. After washing, sera from individual rabbits were serially diluted and incubated at 37<sup>0</sup>C for 2 h. After washing anti-rabbit HRP conjugated IgG (1:10000) (Sigma Aldrich) was added, incubated for 1 h, washed and reactivity visualized by the addition of TMB substrate. The reaction was stopped by adding 2M sulphuric acid. The absorbance values were recorded at 492 nm. The cut off value calculated as double fold of O.D mean negative sera.

**Statistical analysis:** Experimental data were analyzed using ANOVA. Significant difference (LSD) and X Chi post-hoc (SPSS computer program).

## Results

**Purification of PPCatA:** *F. gigantica* Cathepsin proteinases were purified to homogeneity from ES products by gel filtration chromatography. Analysis by SDS-PAGE (under reducing conditions) revealed that the purified preparation contained single proteins of 27.6 KDa, corresponding to cathepsinL1.



**Figure (1): SDS-PAGE of *F.gigantica* PPCat antigen, MW. Molecular weight marker**

**Morphometric study of liver:** Rabbits were killed 12 weeks after challenge and the livers assessed for damage. Table (1) , Control group rabbits were vaccinated with PBS. Two of four control group rabbit livers had the high score of 4, indicating damage between 40% and 80% of the liver surface, while animals vaccinated with PPCat had none or minimal observable damage.

The mean damage score in all of antigen-vaccinated groups was significantly lower than those in the control group.

**Table (1) Mean liver damage scores in infected rabbits.**

Group	Mean liver damage score
PPCat	0.13 ±0.13
ESA	0.26 ± 0.08
SA	0.53 ± 0.17
Control	0.85 ± 0.24

**Parasite burdens and FEC in the animals:** Twelve weeks after the infection with metacercariae of *F.gigantica*, the animals were killed and the parasites in the bile ducts and the liver tissue were counted (Table2). The vaccinated animals in group 1,2 and 3 showed statistical differences (P<0.01) mean reduction in fluke burden of 83, 53 and 39 (P<0.01), respectively. The FEC analysis showed a significant reduction of eggs in the vaccinated groups compared to control group. For group 1, the mean reduction was 100% and for group 2 and 3 it was 50%. Table (3) shows that the liver fluke size and mass are reduced in the vaccinated groups. No significant differences were found between these group and significant differences were found between the length worm in rabbit vaccinated with PPCat and control group.

**Table(2) Feces egg number and fluke burdens in immunized and non-immunized experimentally infected rabbits**

Group	Mean egg	Reduction (%)	Fluke burdens	Reduction (%)
PPCat	0.00 ±0.00	100	2.00 ± 2.31	81.3
ESA	0.25 ± 0.50	50	5.00 ± 1.41	53.49
SA	0.25 ± 0.50	50	6.50 ±1.00	39.53
Control	0.50 ± 0.58	-	10.75 ± 1.71	-

**Table (3) Morphometric analysis**

Group	Mean body length	Mean body width	Mean wet weight
PPCat	0.53±0.92	0.46±0.69	17.52±24.50
ESA	1.03±1.55	0.55±0.89	29.22±52.50
SA	1.68±2.43	0.65±0.96	40.50±79.75
Control	1.41±4.03	0.98±1.98	71.53±85.00

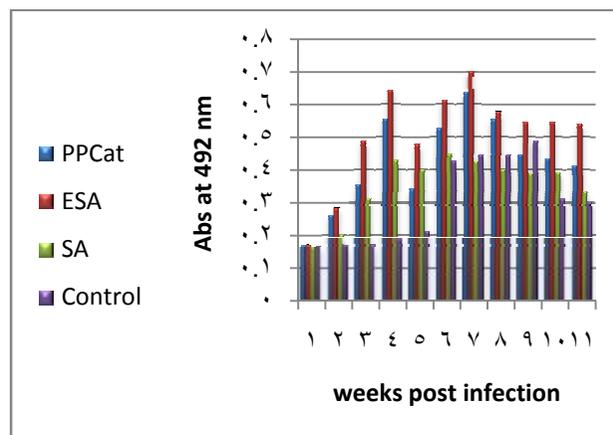
**Viability of eggs recovered from animals:** The viability of eggs recovered from each animal are presented in table (4). Eggs with viability of 80 to 100% were recovered from the gall bladders of all animals in the control group (group 4). The mean viabilities of eggs recovered from animals vaccinated with PPCat (group 1), ESA (group 2) and with SA (group 3) were 100% and

50%, respectively, taking into account that eggs recovered from some animals partially developed but did not embryonated to miracidia. Eggs were not recovered from some vaccinated animal.

**Table (4) Viability of *F.gigantica* eggs recovered from gall bladders of immunized and control rabbits**

Group	Egg viability (%)
PPCat	0
ESA	10
SA	40
Control	80

**ELISA analysis:** The antibody responses of vaccinated and control animals were analyzed by ELISA using PPCat, ESA and SA *F.gigantica* as antigens. Figure(2) showed the mean IgG levels in each group. IgG were observed in all vaccinated animals 1 week after the first immunization. A boosting of the antibody responses was observed in all groups following both second and third immunization. The sera taken before challenge from the control animals did not contain antibodies reactive with an antigen. An increased in the IgG titers in all vaccinated animals was observed within 2 weeks following challenge. Antibodies in all vaccinated groups remained high throughout the infection but decreased at approximately 6 weeks after infection. IgG to antigens in the sera of the control group increased within the first 2 week after infection, which peaked at week 8 and then decreased and then remained constant throughout the subsequent weeks.



**Figure (2) Analysis of IgG responses of vaccinated and control rabbits by ELISA**

## Discussion

Recent work was build in order to develop the effective vaccines for controlling the fasciolosis. The results presented in this study showed that it was possible to induce protective levels against *F.gigantica* in rabbits using crude and purified antigens. The study was evaluated three antigens of *F.gigantica* (SA, ESA and PPCatA) by using vaccination trial in rabbits.

The SA, ESA and PPCatA antigens were chosen for this study basing on their previous successful use against *F.hepatica* and *F.gigantica* (20 ,18 ,21,8, 22). In the present study, we demonstrated the achievement of significant levels of protection against fasciolosis in rabbit by vaccination with antigens.

Accordingly, the *F.gigantica* PPCat antigen defined in this study by SDS-PAGE revealed one band at molecular weight of 27.6 KDa. This result correspond with (23).

Liver fluke size and width, total wet weight of recovered parasites and liver lesion are considered as an important parameters often used to indicate protection against severity of infection and pathology (24, 25, 26). All the antigen vaccinated groups showed significantly reduced liver lesions. All of the antigen-vaccinated groups yielded fewer liver flukes than the control animals, and showed that PPCatA are able to mediate immunity to liver fluke infection.

Current study demonstrated the reduction in FEC (fecal egg count) and fluke burden (anti-fluke effect) in rabbits immunized with tested antigens in comparison with non-immunized control group, the PPCatA immunized group showed the highest reduction in EPG and fluke burdens followed by ESA and SA immunized groups. The antifecundity effects induced by vaccination with the PPCatA are consistent with the similar finding of (27) who vaccinated sheep with mixture of liver fluke cathepsinL proteinases and with those of (11) who showed an almost complete antiembryonation effect in cattle by using CL1 in combination with hemoglobin (Hb). Flukes recovered from vaccinated animals tended to be smaller than those recovered from control, and in particular, in those animals vaccinated with PPCatA.

We speculated that vaccination with antigen would induce antibodies that could interfere with the activity of these antigen and there by block some or all of the above described crucial processes. Suprisingly, the PPCat antigen induce significantly higher level of protection and had greater effects on fluke growth and egg viability than the ESA and SA. Although ESA induce cross-reactive humoral immune responses, ESA may have a better immunoprotective property than PPCat by stimulating higher antibodies. Immunization with ESA

also elicited protective immune responses in rabbit against a liver fluke infection, the ES of many digenetic trematode play vital roles in host parasite interactions including digestion, invasion and immune evasion (28) and at least 60 prominent, ES proteins have been found, with a high number and abundance of protective enzymes, pointing to the central role of ES protecting *Fasciola* from host immune responses (29).

The greatest protection was afforded by a vaccine that contained PPCat, this resulted in a reduction of mean liver burdens (protection) of 81% and some rabbits in ESA and PPCat did not have any detectable liver fluke. These results are comparable with other trials using mice host and cathepsin as vaccine (25,26). The mechanism of protection induced by PPCat vaccine is unknown but may be related to antibody production after vaccination that neutralizes the activity of the proteases, activates cellular effector mechanisms, directly affects the parasite or a combination of these.

In the vaccine trial, there was no significant difference in the egg count between any of the three antigen delivered as vaccines. Gross morphometric examination of the liver showed that lesion were despite the fact that the number of flukes in both groups was quite similar. This result could be due to a lower capacity of larvae to induce hepatic damage in the immunized rabbits or to the random migration of larvae. In some groups, larvae numbers of fluke may have randomly migrated thorough the left hepatic lobe leaving the rest of the liver virtually unaffected, while other animals with lower number of flukes presented numerous scars in both hepatic lobes. This finding may explain the lack of correlation between gross hepatic damage and fluke burdens which was also reported in a previous trial in rabbits (7, 30, 31).

High antibody titers were induced by all vaccine preparations, and these were boosted following the challenge infection. This result confirmed that these antigens were immunogenic (20, 23).

As mentioned, the lowest titre obtained was toward SA, from rabbits vaccinated with ESA and PPCat. This probably reflects the low immunogenicity of SA, and the lack of cross-reactive antibodies induced by the PPCat antigen of the vaccine, PPCat vaccinated groups showed significantly reduced length. As liver fluke body size and width are considered good indicators of fluke development this suggests liver fluke development was retarded in the vaccinated groups, and the severity of the infection was reflected by the reduced liver damage.

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