EXPRESSION OF CATHEPSIN L GENE OF FASCIOLA GIGANTICA IN DIFFERENT DEVELOPMENTAL STAGES

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ABSTRACT

Fascioliasis is one of the most serious parasitic infestations of livestock. It is caused by Fasciola hepatica (Temperate regions) and Fasciola gigantica (Tropical regions).It also has a zoonotic importance. The proteolytic enzyme; cathepsin-L cysteine proteinase (28-28.5 KDa) helps the parasite in tissue invasion, host immune evasion and nutrients intake. This study aimed to check weather Fasciola gigantica flukes depend upon cathepsin-L throughout their life cycle in snail and mammalian hosts or not. For that cathepsin-L gene was investigated in adult and other developmental stages of Fasciola gigantica using semi-quantitative RT-PCR assay. Cathepsin-L protein was detected by using Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE) analysis and detection of cathepsin-L cysteine proteinase antigen in examined stages by Enzyme linked immunotransfer blot (EITB) analysis. Stages of Fasciola gigantica were collected from an experimental life cycle that was completed in our laboratory. The adult worms and eggs were collected from bile ducts and gall bladders, respectively, of slaughtered naturally infected cattle at El-Shohada abattoir, Minufiya Province, Egypt. The results obtained reveled that cathepsin-L gene is expressed in adult and other developmental stages of Fasciola gigantica with a molecular weight of 28-28.5 kDa. These results are promising for developing an effective and reliable method for early diagnosis of Fasciola gigantica where cysteine proteinase enzyme is present in adult and other developmental stages with molecular weight at 28-28.5 kDa and its gene is fully expressed in adult and other developmental stages.

Key words: *Fasciola gigantica, Cathepsin L, mRNA, RT-PCR, EITB.*

INTRODUCTION

Fasciolosis is a hepatic parasitic infection caused by F. hepatica or F. gigantica that affects numerous mammalian species, mainly ruminants and occasionally human, in several countries of Europe, Asia, America and Africa, particularly in Egypt. The economic significance of fasciolosis is mainly due to either

direct losses following decreased growth rate, low milking capacity and the confiscation of altered livers in slaughterhouse **(Gajewska et al., 2005)** or indirect losses due to the interference with the reproductive efficiency as well as retardation in the growth of young animals **(Heath et al., 1997)**.

In human, the presence of adult fluke in the bile ducts causes a variety of symptoms such as malaise, long standing fever, and weight loss, pain under the right costal margin, eosinophilia and anaemia caused by feeding on the host blood **(Soulsby, 1982)**.

Fasciola gigantica in Egypt was reported by **Haseeb et al. (2002)** who stated that it is a serious disease affecting different categories of animals as sheep, goats, cattle buffaloes, horses, rabbits, donkeys and camels, as well as man. The world health organization reviewed the importance of human fascioliasis as a public health disease; this is due to the number of human cases recorded (about 2.4 million people) with liver flukes worldwide **(Mas-Coma et al., 1999).**

Bentancor et al. (2002) stated that cathepsin L proteinases (CL1 and CL2), are the major components of Fasciola hepatica excretion / secretion products (E/S) and considered potential antigens of a vaccine against fascioliasis. They analysed the humoral response elicited by CL1 and CL2 in rats immunized with the enzymes or infected with F. hepatica, by examining specific IgE and IgG subclass dynamics. Moreover, **Kuk et al. (2005)** said that cathepsin L1 (CatL1) is one of the major molecules in the excretory-secretory products of Fasciola hepatica and was secreted by all stages of the developing parasite.

Early diagnosis is not possible because eggs don't appear in feces until flukes reach maturity, usually between 8 and 12 weeks after infection. The question was developed about possibility of usage of protein antigen of F. gigantica isolate in the immunodiagnosis of the disease in human and animal which will be simple, rapid and cheap, early as possible (2-4 weeks post infection), more sensitive and more specific. Also, recent results from several laboratories have demonstrated that animals can be significantly protected against infection by vaccination with defined native Fasciola antigens, which were isolated from excretory-secretory products of the parasite, cathepsin L and haemoglobin **(Dalton et al., 1996)** or by fatty acid binding protein and glutathione **(Spithill and Dalton, 1998)**. A part from reducing fluke burdens, such vaccines can elicit a concurrent reduction in parasite egg production. So there is need to develop cost-effective, environmentally safe and sustainable strategies, such as vaccination, for control of this disease.

MATERIALS AND METHODS

Lymnaea caillaudi snails were collected from Abo-Rwash, Giza Province and reared as previously described by El-Gindy and El-Gindy, 1964. Adult Fasciola gigantica were collected from the common bile duct of naturally infected cattle slaughtered in El-Shohada abattoir in Menofia Province. The adult flukes were identified, washed three times with 0.9% NaCl solution and frozen in 0.9% NaCl solution at (-40^{o}C) until used for RT-PCR. Eggs of Fasciola gigantica were collected from the gall bladders of naturally infected cattle slaughtered in the abattoir and

prepared for RT-PCR according to the method described by **Amer (1996)**.

For Embryonation of eggs, it must be as fresh as possible, so the eggs of Fasciola gigantica were collected from the gall bladders of naturally infected cattle slaughtered in El-Waraq abattoir in Giza province near to the laboratory. These eggs were let to embryonat for Production of Miracidia in the lab according to the method listed by **(Boray, 1964)**. This miracidia will be used for infection of snails.

Infection of Snails:

Infection processes were carried out as previously described by Abdel Ghani, 1964. Briefly, Lymnaea caillaudi snails were exposed individually to the required dose of freshly hatched miracidia by using 25 x 30 cm transparent plastic plates containing 108 finger-like cylindrical depression. By using a pasture pipette, the required miracidial dose (approximately 1- 3 miracidia / snail) was transferred in a small drop of water to every chamber. The snails were added directly (one snail / chamber). The chambers were filled with dechlorinated water. Then, the plates were covered by a transparent perforated plastic cover, and exposed to the miracidia for 12 hours. After the exposure period, the snails were transferred to the plastic aquaria and labeled with the infection date.

Collection of Sporocyst, Rediae and Cercariae:

At 10^{th}-18^{th} days post snails infection, Sporocysts were collected by removing the shells of the dead snails, suspended in distilled water and stored at - 40^{o}C until used. At 25^{th} day post infection, rediae were collected by removing the shells of the dead snails, suspended in distilled water and stored at - 40^{o}C until used. Before the suspected day of shedding (i.e., day 29), the shell of the dead snails were removed and the cercariae were collected in distilled water. Also, after shedding, cercariae have been collected on aluminium foil that fixed on the wall of the aquaria at the level of the water. The collected cercaria was suspended in distilled water and stored at - 40^{o}C until used.

Preparation of total RNA:

The total RNA was extracted using QIAGEN, RNA extraction kit (QIAGEN,, Germany). according to the manufacturer's instructions.

Table (1): Sequence, position primers of CL-1 gene and product size.

Primer	Sequence	Position	Product Size
Forward (5 – 3)	ATGACGATTTGTGGCATGAA	50-69	250 bp
Reverse (5 – 3)	GGGATACCGCGTGAGAGTAA	280-299	

The selected CL-1, gene primer manufactured by (Metabion, Inc., Germany).

Primer Design:

Primers were designed as shown in Table1, based on published nucleotides sequences (gene bank accession number u62288).

Reverse Transcriptase / Polymerase Chain Reaction (RT-PCR):

3 _g RNA were reverse transcriped in thermal cycler at 42^{o}C for 30 minutes by using Ready-Go RT-PCR kit, (Amersham, USA): A pellet that contains all ingredients necessary for RT-PCR assay including reverse transcriptase, RT buffer, Taq polymerase, 10x buffer and deoxynucleotide triphosphate mix (dNTPs). The pellet was resuspended for direct applying RT-PCR assay. The PCR continued by heating the samples at 95^{o}C for 5 minutes for initial denaturation. The PCR will performed for 40 cycles each one composed from 3 different step; the 1st is denaturation at 92^{o}C for 1 minute, anealing step at 50^{o}C for 1 minute and Extension step at 72^{o}C for 1 minute. The RT- PCR product was detected using agarose gel electrophoresis according to **Sambrook et al. (1989)**.

The excretory- secretory (E/S) Products Antigen of Adult Fasciola gigantica were prepared according to Santiago de **Weil and Hillyer (1986)**. While the different Developmental Stages Antigens were done as previously described by **Amer (1996)**. The total Protein concentrations were determined by Lowry method **(Lowry et al. 1951)**. Each sample containing 10 μg of protein was dissolved in sample buffer in ratio of 1: 2 and boiled in boiling water bath for 2 mintutes. Samples along with prestained broad range molecular weight protein marker (Bio- Lab Company) were then subjected to 10% SDS-PAGE. The gel was stained with Commassie blue stain. Protein bands were scanned, and analyzed by densitometer to determine the molecular weights.

Western blot analysis :

For western blotting, the protein samples were subjected to 10% SDS-PAGE then electro-blotted onto a nitrocellulose membrane according to the modified technique by **Towbin et al. (1979)**. The nitrocellulose membrane was soaked in a blocking buffer for 2 hours and washed in washing buffer 2 times for 5 minutes / each. Serum solutions were diluted in 5% BSA in 0.3 % PBST then , the nitrocellulose membrane was exposed to the diluted sera for one hour and washed 2-3 times for 5 minutes / each in washing buffer, then exposed to peroxidase labeled antibody diluted in 5% BSA in 0.3% PBST for one hours. The membrane was washed 2-3 in washing buffer and exposed to the substrate for 30 minutes. The membrane was then rinsed thoroughly with distilled water to stop the reaction. The reaction was read by Gel pro-analyzer 3.1 (Multimedia Co., USA).

Preparation of Hyper Immune Sera from Rabbits:

Six male 1.5-2 Kilogram New Zealand rabbits were divided into 2 groups (three rabbits for each). Group (A): Immunized with purified cathepsin antigen (supplied by Biotechnology Center for Research and Services (BCRS), Cairo University, Egypt).Group (B): Injected with adjuvant alone as a control group.

Immunization Protocol: (According to the method described by Langley and Hillyer, 1989):-

In Group (A): The animals were immu-

nized by purified cathepsin-L antigen with Freund's adjuvant in ratio of 1:1 (injected S/C in two sites above the shoulder at three doses), the 1st injection at zero day was 200 _g protein of the prepared antigen per animal which were obtained from 200 _l of the soluble extract with Freund's complete adjuvant (The complete Freund`s adjuvant containing attenuated strains of Mycobacterium which lead to non specific immune response). The rabbits then injected with 100 _g protein per animal (which were obtained from 100 _l of the soluble extract) with Freund`s incomplete adjuvant at 14 and 21 day from beginning of the experiment.

In Group (B): The animals were injected with a dose of Freund`s complete adjuvant alone at zero day S/C in Two site above the shoulder, then the second and third doses were with Freund`s incomplete adjuvant at 14 and 21 day from the beginning of the experiment, respectively. Blood samples were collected at 28th day post immunization. Serum was prepared according to **Rogan (1996)**.

RESULTS

Gene expression analysis:

Cathepsin L mRNA expression in Fasciola gigantica eggs, meracidea,sporocysts, rediae ,cercariae and adult fluke. As showen in fig. 1, a band of 250 bp was detected in the egg and other developmental stages. This size is identical to that of the positive control; Fasciola gigantica DNA.

After detection of cathepsin L mRNA expression by RT-PCR, We checked Fasciola gigantica adult E/S and developmental stages proteins by SDS-PAGE. The results showed expression of 28.5 K Da protein in all tested samples (Fig: 2A&B, table 2). This indicates the presence of that protein throughout the life cycle of Fasciola gigantica .

Enzyme linked immunotransfer blot analysis (EITB):

To check weather the mRNA expression of cathepthin L is supported by protein expression, EITB analysis was applied on protein sample from adult Fasciola gigantica (E/S)products and other developmental stages. Fig.3A &B, table3 Showed that cathepsin L protein was detected as a single protein band of 28 K Da in all tested samples.

DISCUSSION

Several authors have been involved in studying the proteases during development of parasites such as the serine proteases from larvae of Heliothis virescens **(Johnston et al., 1995)**, the neutral proteases of the three developmental stages of Shistosoma mansoni **(Auriault et al., 1982)**. Enzymatic investigations have shown that Fasciola hepatica cathepsin L is expressed in different developmental stages **(Carmona et al., 1993; Hawthorne et al., 2000; Muharsini et al., 2000 and Harmsen et al., 2004)**.

Concerning Cathepsin-L mRNA Expression in Adult and Different Developmental Stages of Fasciola gigantica:

Our knowledge indicated that there is no similar study conducted on Fasciola gigantica.this motivated us to perform this work on Fasciola gigantica. The present study showed the expression of cathepsin-L1 cysteine proteinase gene in adult and other developmental stages of Fasciola gigantica (eggs, miracidiae, sporocysts, rediae and cercariae) at the expected band size of 250 bp using RT-PCR.

These results are in accordance with that of **Tort et al. 1999** and **Grams et al. 2001; Law et al. 2003** and **Mohamed et al., 2005** on Fasciola hepatica.

Regarding Cathepsin L Protein Expression In adult and different developmental Stages SDS-PAGE analysis;

SDS-PAGE results showed the presence of the 28 KDa protein band within the proteins samples of the adult E/S products and the other developmental stages. These results are going with those of Smith et al. 1993b and Dowd et al., 1994 who proved that the major proteins secreted by adult liver flukes were the 27-kDa cathepsin L1 and 29.5-kDa cathepsin L2 proteinases. Moreover, our findings are in accordance with that of **(Fagbemi and Hillyer 1992; Smith et al., 1993b; Kuk et al., 2005; and Sriveny et al., 2006)**.

EITB analysis:

EITB analysis in this study showed the presence of a band of cathepsin L1 cysteine proteinase at 28 KDa, in adult and other developmental stages of Fasciola gigantica (eggs, miracidiae, sporocysts, rediae and cercariae).This indicating the presence of cathepsin L1 proteins in these stages.

Amounts of cathepsin L proteins from EITB analysis differed from stage to stage. In eggs the amount was the lowest (16.102 ± g) then we noticed increase in the cathepsin L protein in the miracidia (21.878 ± g) this may explain the role of the enzyme in penetration of the snail tissues by miracidia. The amount decreased in sporocysts (20.826 ± g) then increased again in rediae and cercariae (24.765 _g, 24.069 ± g, respectively). This increase may be due to the demand of cercariae for cathepsin L for facilitating the excystation of encysted metacercariae and penetration of the final host intestinal wall.

Another definite increase in the amount of cathepsin L proteins in E/S products of adult Fasciola gigantica was noticed (34.018 ± g). This was the highest amount in all stages. This may explain the great role of that enzyme in Fasciola gigantica adult stage either in parasite nutrition, tissue penetration and final host immune evasion.

The results obtained in this study came in agreement with **Gorman et al., 2000; Ortiz, 2000; Bossaert et al., 2000. Also Dixit et al. 2002** discovered that the major antigenic fraction of Fasciola hepatica ES antigen 20-30 KDa was recognized specifically by sera from F. hepatica infected calves as early as 6-8 weeks PI.

We can conclude that Fasciola gigantica parasite is completely depend upon cathepsin-L cysteine proteinases along its whole life cycle either in snail and in mammalian host. This means that cathepsin-L cysteine proteinase is an immunodominant antigen in Fasciola gigantica life cycle. This study predicts that cathepsin-L cysteine proteinases of Fasciola gigantica may be the most promising antigen for immunodiagnosis of human and animal fascioliasis and for protection against the disease through vaccination strategies.

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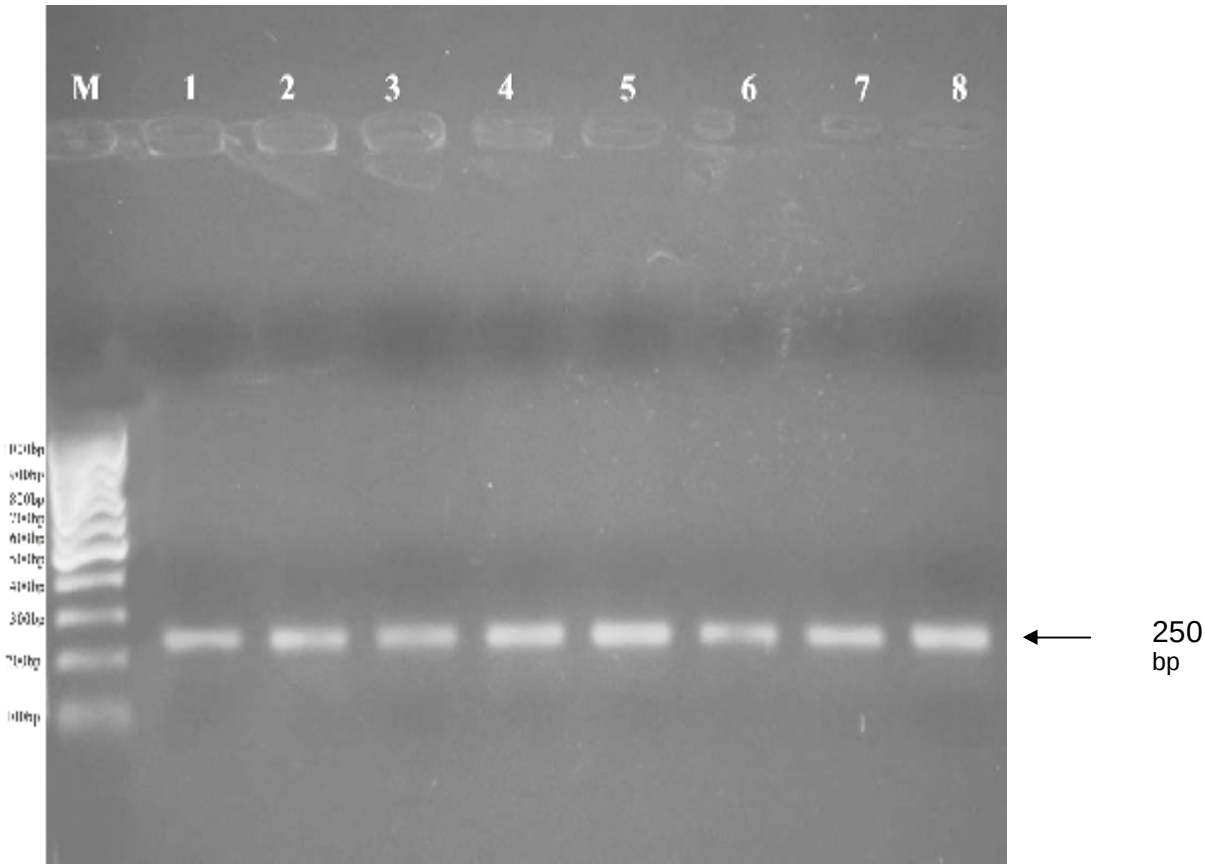
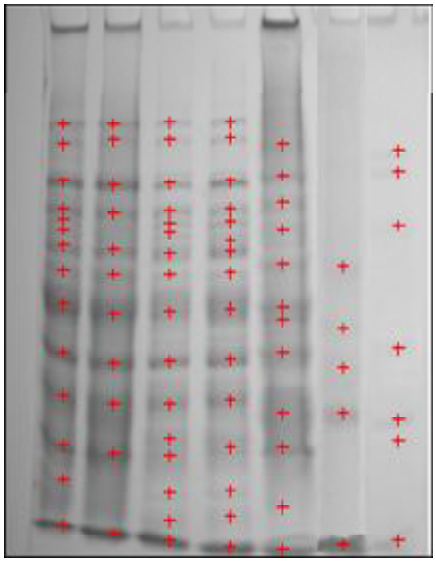


Figure (1):

Cathepsin-L1 Gene Expression Analysis Depending On Detection Of mRNA By RT-PCR Amplification Assay.

Lane (1): Eggs. Lane (2): Miracidia. Lane (3): Sporocysts. Lane (4): Rediae. Lane (5): Cercariae. Lane (6): Adult. Lane (7): Adult. Lane (8): positive control adult Fasciola gigantica

(A)



(A)

Figure (2): Coomassie blue stained SDS- PAGE of *Fasciola gigantica* Adult E/S products and developmental stages proteins. (A) Coomassie blue stained SDS- PAGE gel and (B) Photo of Gel pro-analyzer.

E: Egg M: Miracidia S: Sporocysts R: Rediae C: Cercariae
E/S: Excretory-Secretory products Ma: Marker

(B)

Table (2): Molecular weights and amounts of protein bands appeared in SDS-PAGE analysis of adult *Fasciola gigantica* E/S and developmental stages proteins.

	Egg		Miraci		Sporo		Redia		Cercaria		E/S		Marker	
Lanes:	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5		Lane 6		Lane 7	
Bands	(mol.w.)	(amount)*	(mol.w.)	(amount)	(mol.w.)	(amount)	(mol.w.)	(amount)	(mol.w.)	(amount)	(mol.w.)	(amount)	(mol.w.)	(amoun
1	140.18	1.5938	140.18	3.2826	142.04	1.4415	142.04	1.0021	121.58	13.033	56.505	8.2491	116	2.0841
2	121.58	2.2514	125.3	6.0042	125.3	0.86246	127.16	1.2831	95	4.3766	42.447	2.9478	97.4	2.1872
3	91.4	4.7132	89	4.9755	90.2	2.28.587	91.4	4.3843	79.4	2.9423	35.191	3.4188	66.2	7.6514
4	75.8	3.6782	73.4	3.5891	74.6	2.3651	75.8	2.4018	65.231	3.0793	28.5	16.811	37.6	3.6273
5	69.8	10.217	60.383	3.8009	67.4	4.0279	68.6	4.8514	56.99	3.1326	13.813	40.481	28.5	0
6	65.231	11.796	54.566	3.4928.5	64.261	5.3688	62.322	7.4531	47.28.55	6.996			18.4	26.986
7	61.353	4.4124	45.841	14.102	59.414	4.5673	59.898	7.3032	44.386	11.837			14	32.453
8	55.536	2.8916	35.726	7.7286	54.566	1.5964	55.051	1.8133	37.065	8.9441				
9	47.78	15.352	28.5	16.137	46.325	14.084	46.81	15.43	28.5	19.443				
10	37.065	9.592	17.838	15.048	35.994	7.9771	36.262	10.438	18.119	34.211				
11	28.5	13.319	14.374	7.2178	28.5	10.846	28.5	13.421	15.498	0.85867				
12	18.213	13.28.55			19.318	6.7215	18.119	13.763	13.626	7.8271				
13	16.715	9.0954			17.745	17.492	16.153	3.4368						
14	14.749	8.0038			16.06	3.3416	15.123	67.343						
15					14.936	5.6307	13.719	11.28.57						
16					14	15.6								

*Amounts of protein bands in

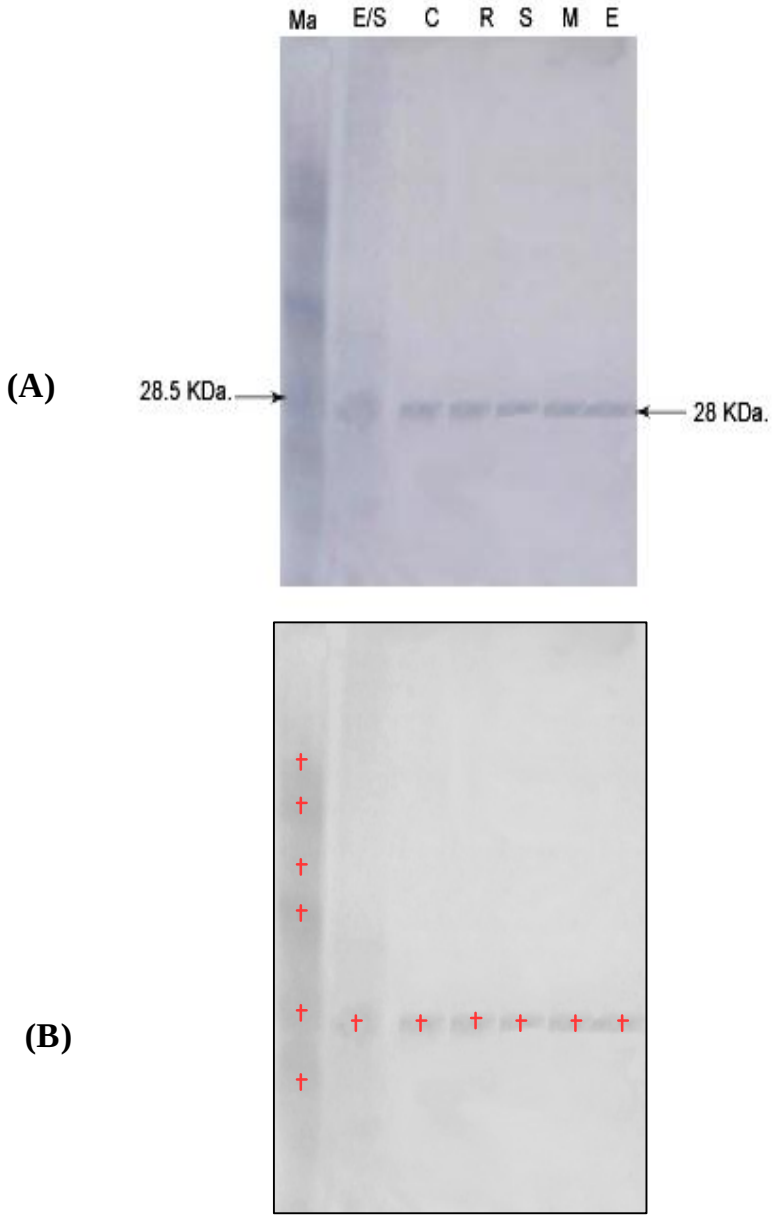


Figure (3): Immunoblotting of adult *Fasciola gigantica* E/S and different developmental stages antigens (eggs, miracidiae, sporocysts, rediae, cercariae) with cathepsin L injected rabbit serum.
(A) Detection of antigens on nitrocellulose sheet.
(B) Photo of Gel pro-analyzer.
E: Egg M: Miracidia S: Sporocysts R: Rediae C: Cercariae
E/S: Excretory-Secretory products Ma: Marker

Table (5): Amounts of cathepsin L cysteine proteinase in adult *Fasciola gigantica* E/S and different developmental stages' antig (eggs, miracidiae, sporocysts, rediae, cercariae) as revealed by immunoblotting using cathepsin L injected rabbit serum.

*Amounts of protein bands in µg.

	Marker		E/S		Cercaria		Redia		Sporo.		Miracid.		Egg
Lane	Lane 1		Lane 2		Lane 3		Lane 4		Lane 5		Lane 6		Lane 7
Band	mol.w.	(amount) *	mol.w	(amount)	mol.w	(amount)	mol.w	(amount)	mol.w	(amount)	mol.w	(amount)	mol.w
1	116	8.1562	28	34.018	28	24.069	28	24.765	28	20.826	28	21.878	28
2	97.4	28.569											
3	66.2	35.661											
4	37.6	20.305											
5	28.5	33.932											
6	19.031	98.712											

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الملخص العربى

دراسات جزيئية على كاثيبثن چين فى الديدان الكبدية

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تعتمد الديدان الكبدية على بعض المواد مثل الإنزيمات الهاضمة ومضادات الأكسدة فى إختراقها لجسم الإنسان والحيوان خصوصاً عند اختراقها الكبد وذلك لإتمـام نموها والبدء فى وضع البويضات لاستمرار دورة حياتها ، وأهم هذه المواد هو الكاثيبثن وهو من الإنزيمات الهاضمة للبروتين سواء الموجودة فى المواد الغذائية داخل الجسم أو البروتين المكون لأنسجة الجسم مثل الغشاء البريتونى أو الكبد، لذا قد ركزت الدراسة على تحديد ما إن كانت الديدان الكبدية العملاقة تعتمد على إنزيم الكاثيبثن خلال فترة حياتها بأكملها بداية من البويضات مروراً بالأطوار النامية الموجودة فى العائل الوسيدط (القواقع) من نوع Lymneae caillaudi ، أو الطور البالغ الموجود فى العائل النهائى (الإنسان والحيوانات الثديية) ، واشتملت الدراسة على عمل دورة حياة تجريبية داخل المعمل وتم تجميع الأطوار النامية من خلال هذه الدورة، وتم أيضاً تجميع عينات البويضات والطور البالغ للديدان الكبدية العملاقة بعد تصنيفها من أكباد الأبقار المصابة طبيعياً المذبوحة داخل مجزر الشهداء - بمحافظة المنوفية، ووجدنا أن چين الكاثيبثن يتم استنساخة وترجمته لتخليق الإنزيم فى جميع الأطوار النامية والطور البالغ وتم فصل أنتجينات جميع الأطوار النامية وأنتيجينات إفرازات وإخراجات الطور البالغ للديدان الكبدية العملاقة بواسطة إجراء إختبار التمرير الكهربى SDS-PAGE وكانت النتيجة هى وجود الحزمة البروتينية ذات الوزن الجزيئى 28.5 كيلو دالتون التى تعبر عن هذا الإنزيم، أيضاً تم إجراء تفاعل النقل المناعى EITB وأوضحت النتائج حدوث التفاعل المناعى لكل الأطوار النامية والطور البالغ عند الحزمة البروتينية ذات الوزن الجزيئى ٢٨ كيلو دالتون المعبرة عن الوزن الجزيئى لإنزيم الكاثيثن، وبذلك تم إثبات أن إنزيم الكاثيثن موجود بالأطوار النامية والطور البالغ، وأن الچين المسئول عنه يستنسخ ويترجم إلى هذا الإنزيم خلال هذه الأطوار النامية والطور البالغ للديدان الكبدية العملاقة، بمعنى أن الديدان الكبدية العملاقة تعتمد إعتماداً كلياً على هذا الإنزيم طوال دورة حياتها بأكملها.