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DEVELOPMENT OF FASCIOLA GIGANTICA EXPERIMENTALLY IN EGYPTIAN RABBITS

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Abstract

After infection of three groups of rabbits by 50, 25 and 10 *F. gigantica* encysted metacercariae (EMC) for each animal, a rabbit appears as an unsuitable host for the development of this species of flukes. Rabbits need special precautions to help the worms to reach maturity before death of the infected animals. The juveniles wander in the liver tissue away from the main bile ducts, and may accidentally emerge back to the peritoneal cavity and can remain there or return back by chance to the liver. Survival of the infected rabbits, the degree of pathogenisty produced and the number of extracted worms are affected by the dose of the EMC and the general health conditions of the rabbit. Infective dose (ID) of 50 EMC led to mortalities from 28th to 58th day post infection (dpi.). This was accompanied by detection of flukes in the peritoneal cavity, and the anterior cones of several worms were found penetrating the liver toward the abdominal cavity. At 58th dpi, dead animals showed marked liver cirrhosis and adhesions with the surrounding organs. Decreasing the ID to 25 EMC increased the rabbits survived till 74th dpi. The liver looked enlarged and juveniles successfully reached the main bile ducts.
Successful fluke development, with low adverse changes in the liver appearance was recorded in the group infected by 10 EMC, and the animals survived till 98th dpi. The problem, at this low ID, was the decreasing in the infection rate in the exposed rabbits to 37.5%. In all groups, using ELISA technique, IgM & IgG anti-*Fasciola* antibodies (AFAb) increased gradually in sera. Higher titters were recorded in the group infected by 25 EMC. Prolonged migration and retardation of *F. gigantica* growth in the infected rabbits associated with either a high or a low titters. this means that the produced AFAb lack the capacity to mediate responses that can influence parasite survival. So failure of development of *F. glgantlca* growth in rabbit appears to be related mainly to its innate resistance against this *Fasciola sp.*
Key words: *F. gigantica*, Rabbit, Pathology, Immunology.

Introduction

Using of small non-expensive animal model was considered as an important cost benefit point in screening of new drugs or even carrying trial of different dose of vaccine. Rabbits are commonly used laboratory animals. Several authors described the successful experimental infection of rabbits with *F. hepatica* and development of worms up to egg laying (Sherif, *et al*, 2001, Adedokun and Fagbemi, 2001; Abdolahi and Shahriari 2016). Mango *et al*. (1972) reported that East African *F. gigantica* couldn't reach sexual maturity in mice, rats, hamsters, Guinea pigs and rabbits infected by dose of 5 & 25 EMC/rabbit, with rabbits and rats the least susceptible ones, they added that *F. gigantica* in rabbits were dead and calcified by immune protection. Gerber *et al.* (1974) inoculated 30 rabbits with *F. gigantica* 20 metacercariae, found that on the 21st day post infection about 50% were infected by young flukes. After 10-12 weeks the flukes developed in length but did not reach fertility but, infected rabbits suffered from disturbances of general health status, sever necrotic liver damage and died. They concluded that rabbits were not suitable model for *F. gigantica* but a useful animal to check the infectivity of metacercariae and to screen anthelmintics against flukes young stages. On the other hand, Smyth (1994) found that

after infection of rats by *F. hepatica,* the rat's antibody-mediated cytotoxicity responses killed larval stages and young flukes as they migrate across the body cavity and through the liver. He added that sheep also, responded immunologically and produced anti-fluke antibodies, but these antibodies appeared to lack the capacity to mediate responses that can influence parasite survival. But, with *F. gigantica,* there was no data about its development till egg deposition in rabbit except three Egyptian papers. These were Ezzat and Abdel Ghani (1960), Hassanain *et al.* (1990) and EI-Sayad (1997) who infected rabbits by 30 EMC of *F. gigantica* and detected mature flukes that gave up to 180 EPG (egg/gm feces).

Around this wide deference between the local and foreign studies as regard susceptibility of rabbits to *F. gigantica,* and the real need of a small economic animal model for accurate evaluation of new fasciolicidal drug or vaccine, or even trials of different doses of vaccine, the present study aimed to evaluate rabbit as a non-expensive laboratory animal model for our native *F. gigantica.*

The study aimed to clarify the condition under which rabbits can survive till *F. gigantica* reached sexual maturity. This was done by infecting rabbits with different doses of EMC and evaluated the effect of the dose on the fluke survival in the infected rabbit, changes in liver, fluke age and size. Also to clarify the role of antibodies produced post infection on retardation of juvenile growth and migration outside the liver or this related to rabbit innate resistance.

Material and Methods

This study was designed and approved by Faculty of Veterinary Medicine, Cairo University Ethics Committee and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki, and that of Faculty of Medicine, Al-Azhar University. The study was carried out from April to September 2017.

Preparation of EMC: *F. gigantica* EMC were collected from laboratory breed *Lymnaea natalensis* 25-28 dpi of each snail by 4-5 miracidia under laboratory conditions. These miracidia hatched after incubation of *F. gigantica* eggs isolated from gall bladder of freshly slaughtered *F. gigantica* infected buffaloes (EI-Bahy *et al,* 2014). EMC were collected and stored on cellophane sheet at 4°C till used after 5-8 days.

Infection: Thirty-two white New Zealand rabbits (1.5-2kg each) divided into 4 groups of eight rabbits each. Each rabbit in GI was infected by 50 *F. gigantica* 8 days old EMC, rabbits in GII were infected each by 25 EMC, and in GIII were infected by 10 EMC each. GIV were kept as non-infected control. Before infection, rabbits were fastened for 24 h, ID of EMC were placed on a cellophane sheet, enclosed inside lettuce and were put over the roots of rabbits' tongues. The mouth was kept closed by hand and observed for another half an hour to ensue completed swallowing without regurgitation. Then rabbits were maintained on dry food and water until the end of the experiment.

Slaughtering time and Post-mortem examination (PM): The infected rabbits were kept to live as long as possible. PM examination was applied on any of one died naturally at any post infection time. At 120dpi (Adedokum and Fagbemi, 2001) all rabbits were slaughtered, abdominal cavities were dissected out, degree of adhesions and colour of peritoneal fluid were recorded. Viscera and liver surfaces were macroscopically inspected by a hand lens (x10). Liver was extracted out to a Petri-dish with warm distilled water (35-37^{o}C). Abdominal cavity and other visceral surface were washed three times with warm water, which was collected, sieved and examined for any worms or part of them. Rabbits' liver surfaces were inspected for necrotic migratory furrows, if present; they were dissected to extract the juvenile flukes (EI-Bahy *et al,* 2014). Liver was then sliced into small pieces in warm water and examined under a binuclear microscope. All worms or anterior pieces of wor-

ms recovered from liver or peritoneum were collected, counted, compressed, fixed, stained and mounted in Canada balsam (Pritchard and Kruse, 1982). Measurements were taken by micrometre eye-piece, minimum and maximum values were recorded separately.

Serological study: ELISA was applied to evaluate the antibody response to *F. gigantica* experimental infection in rabbits in relation to dose and time PI.

Serum samples: Blood samples (1-3ml) were collected from ear vein of each rabbit (infected and control) at 1, 2, 4, 6, 8, & 10 weeks PI., left to clot and sera were stored.

Antigen used: *F. gigantica* excretory secretary antigens (ES) was prepared (Abdolahi and Shahriari, 2016). Fresh worms extracted from an infected buffalo gall bladder and washed 3 times in PBS, were incubated in PBS (pH 7.2, 1worm/5ml) for 3 hrs at 37°C. The supernatant was isolated by cool centrifugation at 10000 rpm for one hr. Antigenic protein content was determined after Lowry *et al.* (1951), allocated into 1ml vials and stored at -20^{o}C until needed.

Reference control sera (rabbit hyperimmune sera, HIRS): Three (2 months old) white New Zealand rabbits, parasite-free, maintained under specific pathogen-free condition with suitable humidity and temperature, at Department of Parasitology, Veterinary Medicine, Cairo University. HIRS were prepared (Tang *et al*, 2015) with modification. Pre-immune sera were collected before immunizations (as negative control). Rabbits were subcutaneously immunized with 1.2mg protein of ES Ag, emulsified 1:1 (v/v) with mineral oil as adjuvant, followed by three boost immunizations at 2-week intervals with 0.4mg protein with same adjuvant. Two weeks after the last immunization, rabbits were bled from the ear vein for serum collection. The sera were stored at -20°C until used as positive control.

ELISA evaluated the antibodies against *F. gigantica* developed in rabbit in relation to dose and time post infection (PI.) after Liu *et al*. (2015) with little modification. Each plate was coated over night with antigens at optimal dilution 4ug/ml in coating buffer (optimal dilutions of various reagents were determined by checkerboard titration.). After washing, sera and reference control hyperimmune sera at 1:100 dilutions were added (100ul/well), incubation at 37°C for 90 minutes, and plate was washed and goat anti-rabbit IgM & IgG horseradish peroxidase conjugate (Sigma) were used at 1:2000 dilution. Ortho-phenylenediamidine-OPD was added as 340ug/ml substrate buffer. Absorbance was read at 450nm using a Titerteck multiskan ELISA reader. Positive ELISA optical density= mean value of negative control +2 folds of standard deviation. Sera of 5 rabbits on same day PI and 5 controls were tested separately, two wells for each. Mean of each & five rabbits were recorded as one value. Positive ELISA was compared with control on same time PI (Liu *et al*, 2015).

Results

Infection of 8 rabbits each by 50 *F. gigantica* EMC GI showed a marked dangerous effect on liver and general health conditions causing death within 58 d.p.i. P.M. examination of two rabbits died at 28th & 30th d.p.i. showed liver surface creamy colour with pink rounded spots 1-2mm in diameter and short streaks up to 7x1-2mm specially on right lobe periphery close to duodenum. Anterior cones of two worms penetrated liver surface towards abdominal cavity. Extra-hepatic, bile ducts were normal without gross peritonitis on surface. Two small worms of 3.5x1.0mm & 5x1.5 mm were detected in peritoneal wash of rabbit died at 30th d.p.i.

Liver maceration after that gave 8 & 5 worms little longer than that of peritoneum (6.4-7.6x1.0-1.6mm). The rest of the infected rabbits died at 56th d.p.i. (2), 57th d.p.i. (one) and 58th d.p.i.. (3). During these 3 days, 19 juvenile flukes were extracted from liver (size ranged from 13.3-14.6x1.2-1.6 mm) and 10 worms were in the peritoneal wash (size ranged from 9-11x1-1.4mm) The livers were enlarged firm in consistency, irregular surface, with a thick superficial co-

vering layer of firmly adherent fibrin, whitish in colour connecting liver to surrounding organs. This layer caused firmly adhesion between the liver lobes, unusual pale colour, grossly enlarged (79gm) and firm in consistency. Slaughtering of two controls on same days showed liver normal smooth glistening with weight of 54 gm.

Peritoneal cavities of infected rabbits died at 56th to 58th d.p.i. showed whitish fibrous substances with peritoneal fluid in-between. Rabbits were severely emaciated. Internal organs and muscles were pale in colour with poorness symptoms. Gall bladder was not grossly enlarged but bile was dark brown in colour. Some worms were more superficially at the periphery of the lobs clearing either migration to or from peritoneal cavity with low sensitivity towards bile ducts.

With decreasing EMC dose to 25/rabbit was the time of first mortality delayed to 58th d.p.i. (3), then at 73th d.ph.i. (1) but 4 died at 74th d.p.i. Those died at 85th d.p.i. didn't have severe inflammation or adhesion recorded at same time in G1. Liver was little enlarged, with nearly smooth surface. Three worms were found in peritoneal wash and 12 were extracted from liver tissue near surface lesion. One rabbit died at 73rd d.p.i. showed marked lesion of worm penetration on liver surface but none was extracted after maceration. At 74th d.p.i. four rabbits died from infection were emaciated and weak on day 50th p.i.

The liver enlarged with smooth surface (68 gm), capsule was transparent with neither rough thick fibrin on surface nor adhesion with surrounding organ. Fourteen worms were extracted livers, with length varied from 14.5-16.5x2-3mm. Those extracted from the main bile duct near the hilus region of 2/rabbits were in peritoneal wash, but none in peritoneal cavity. The worms were smaller (12.5-14.5x2-3mm). Two controls slaughtered at 74th day had normal smooth brownish liver with mean weight of 56gm. Details were given in tables (1, 2, & 3) and figures (1 & 2).

Table 1: Infection and frequency of mortality in rabbits infected by different doses of EMC:

Infected Rabbits	Time of death or slaughtered	Dead rabbits	%	Infected rabbits	% infected
G1 50 EMC	28th dpi	1	12.5	8/8	100%
	30th dpi	1	12.5		
	56th dpi	2	25.0		
	57th dpi	1	12.5		
	58th dpi	3	37.5		
G2 25 EMC	58th dpi	3	37.5	8/8	100%
	73rd dpi	1	12.5		
	74th dpi	4	50.0		
G3 10 EMC	96th dpi	2	25.0	5/8	62.5%
	98th dpi	3	37.5		
	120th dpi	3*	37.5		

No mortalities or worms extracted from control non infected rabbits slaughtered in corresponding days of mortality of others.

Table 2: Size range of extracted flukes and rate of infection

Rabbit groups	Death or slaughtering tine	From Liver			From Peritoneum			fluke/ rabbit	fluke/ EMC
		No.	Length	Breadth	No.	Length	Breadth		
G1	28th dpi	8	6.4-7.6	1-1.6	-	3.5-5	1-1.5	5.5	11.0%
	30th dpi	5			2				
	56th dpi	9	13.3-14.6	1.2-1.6	3	9-11	1.1-1.4		
	57th dpi	3			1				
	58th dpi	7			6				
GII	58th dpi	12	12.5-14	1.8-2	3	12	2-3	4.125	16.5%
	73rd dpi	0			0				
	74th dpi	14	14.5-16.5	2-3	4	12.5-14.5	2-3		
GIII	96th dpi	7	17.5-23.5	3-4.2	-			3	18.75%
	98th dpi	8	18-25	3-4.2	-				
	120th dpi	0							

In GIII as each one was exposed to 10 EMC/rabbit, they were still surviving for

long time (96-98 d.p.i.) without marked disturbances. Emaciation and weakness appeared on infected rabbits from the day 65th p.i. Three rabbits from this group (37.5%) did not die, did not show marked change in their general health conditions and still alive.On slaughtering them at 120th d.p.i. they appeared to be overcome this dose level after penetration of the juvenile flukes, where very minute lesions were detected on their liver surface without extraction of any worm after maceration of their liver.

The liver of rabbits died at 96 & 98th d.p.i. appeared as that in fig (1-A) , have normal colour, glistening, and consistency. Its mean weight was 57 gram (mean weight of 2 livers from normal non-infested rabbits of similar age was 58 gram). The only lesions detected on their liver surface was a round elevated necrotic points yellowish to brown in colour representing the site of juvenile worms penetration. There was no adhesion or fibrosis between the liver and the surrounding organs that have a normal colour. Two to three worms were extracted from the main bile ducts of each liver. The extracted flukes were larger than before measured 18-26 mm length and 3-4.2 mm breadth, in a good nourished condition. The mounted specimens showed complete development of most internal organs but there was no egg present in worms' uterus to day 98th d.p.i. Only 4 eggs of *Fasciola* species were small elongated, without embryonic cells and measured 110-125x 45u, were detected in examining warm water of a rabbit died at 98th p.i. after liver maceration. No worm was detected in peritoneal wash at PI. The infection rate was 100% in rabbits exposed to *F. gigantica* in dose of 25-50 EMC/rabbit, but was 62.5% with 10 EMC/rabbit. There was a direct relation between of EMC dose increases and early mortalities.

Level of antibodies developed in infected rabbits**:** The level of AFAb (IgM & IgG) using ELISA was estimated in relation to infection by different doses of EMC as described. The data revealed gradually increase in IgM level directly after infection during the first week post infection (wpi), little decreased during the second wpi and then disappeared. Antibody titer reflexed by ELISA/OD was high in rabbits infected by high dose (0.735) than those infected by low dose (0.622) as compared to control (0.023).

IgG against juveniles increased from 2nd w.p.i., at 3rd wpi, value was higher in GI (0.681) than GII (0.677) or GIII (0.598)

ELlSA OD was increased in GI to 0.655 and 0.671 at the 4th and 6th wpi. respectively and did not increase but it decreased to 0.555 after this till end of the observation period which was associated with death of these rabbits at 58th d.p.i. In the contrary, mean ELlSA/OD was increased in the other two groups for 0.733, 0.700, 0.656 and 643 in GII and it reached to 0.811, 0.786, 0.702 & 0.632 in GIII at the weeks 4, 6, 8 & 10 post infections respectively in comparison with GI and control group. The data showed no marked role for the increase in the dose of EMC in causing severe changes in the level of produced anti-*Fasciola* antibodies on the level of present study

Table 3: Change in mean ELISA values in relation to dose of *F. gigantica* EMC and post infection time.

Dose of EMC/ rabbit	Ig	Mean ELISA value for 5 rabbit samples at,						
		1st wpi	2nd wpi	3rd wpi	4th wpi	6th wpi	8th wpi	10th wpi
G.I	IgM	0.735	0.555	0.033	0.023	0.022	0.024	0.022
(50 EMC)	IgG	0.117	0.421	0.681	0.655	0.671	0.644	0.555
G.1I	IgM	0.665	0.523	0.040	0.035	0.030	0.027	0.028
(25 EMC)	IgG	0.115	0.446	0.677	0.733	0.700	0.656	0.643
G.-III	IgM	0.622	0.456	0.030	0.025	0.027	0.022	0.026
(10 EMC)	IgG	0.116	0.567	0.598	0.811	0.786	0.702	0.632
Control	IgM	0.023	0.021	0.026	0.025	0.021	0.025	0.024
(non infected)	IgG	0.011	0.013	0.014	0.012	0.011	0.015	0.014

wpi = weeks post infection

Discussion

Development of *Fasciola* in the vertebrate hosts was affected by multiple factors control the infective dose (ID) and the host innate resistance. The two factors controlled the suspected pathogenicity that occurred in this host (Mango *et al*, 1972). This work developed during continuous observation recorded among others work on *F. gigantica* in rabbits. This agreed with Gerber *et al.* (1974) Adedokun and Fagbemi (2001) and Sherif *et al.* (2001), but differed with Hassanain *et al*. (1990).

Infection of three groups of rabbits by 3 different doses of *F. ggiantica* EMC proved rabbit as a host but with special condition to for maturity before rabbit death. Development of *F. ggiantica* in rabbit affected by EMC, depended on rabbit health condition, innate resistance, management and nutrition post infection. The juveniles that penetrate liver early in their life returned back to peritoneum around 28th dpi., which caused mortality in weak rabbit (Gerber *et al*, 1974).

In the present study, 50 EMC/ rabbit led to death of all rabbits preceded by weakness and emaciation before death, on PM there was liver cirrhosis, and adhesion with surrounding organs. Extraction of juveniles migrated superficial in liver tissues clarified that their migrated without sensitivity requirement or attraction toward bile duct. This disagreed with Adedokum and Fagbemi (2001) who found that flukes in the bile ducts 40 days after infection in sheep. With dose of 25EMC rabbits survived for long time. Their liver enlarged, degree of adhesion was less, with some flukes in peritoneal cavity and liver. With dose to 10 EMC, rabbits survived without disturbances in health conditions for long time, without worms in the peritoneal cavity. This might be due to the fact that juvenile flukes found enough room to migrate into the liver. A dose was suitable for worm development in experimentally rabbits. This agreed with Adedokun and Fagbemi (2001) and Sherif *et al.* (2001) who used low dose (5EMC) to obtain *F. gigantica* in rabbit. But, this disagreed with Hassanain *et al*. (1990) and EI-Sayad (1997). At 5-10 EMC worm reached maturity but infection failed in some animals (Sherif *et al*, 2001). Failure in some rabbits was related to its' innate resistant to capsulation of early juveniles with low chemotactic sensitivity to reach bile ducts. In the present study, rabbit survived for a time with good health condition and low pathogenicity. This clarified presence of natural fascioliasis infection in rabbits (Apt *et al*, 1993). Migration of juvenile flukes to liver led to severe fibrosis and adhesion between liver and surrounding organs from repeated bleeding due to EMC high dose. The repeated penetration to liver due to high dose caused mortality (Srivastava and Singh, 1972).

In the present study, identification of eggs after maceration of one liver showed proved *Fasciola* development under experimental conditions. Also, absence worm or piece of worm with eggs in the uterus might be due to the fact that eggs originated from one early mature worm, destructed during liver maceration with presence of worms' anterior.

In the present study, IgM gradually increased in the 1st wpi, slight decreased in 2nd and then disappeared. IgG increased in 2nd to 4th wpi and then slightly decreased to experimental end. Changes in antibody level were not related to EMC dose used. Mild variations in level might be related to host ability to deal with infection. This agreed with Itagald *et al.* (1989) in cattle using *F. hepatica*, IgG was low in 1st wpi. and then increased from the 4th to 6th wpi.

Decrease of the antibodies' level in GI after the 6th wpi was related to weakness and ultra-ions in rabbits' health conditions. Doses of 10 or 25 EMC stimulated rabbit immune system to produce more antibodies without bad effect on health conditions. Absence of relation between infective dose and level of AFAb showed that rate of fluke development depended on immune system to tolerate the stress on liver infection. This

agreed with Rickard and Howell (1982) who reported the same in sheep. But, Hughes *et al.* (1981) found that antibody-mediated cytotoxic action killed migrating larval stages and young flukes.

No doubt, the worms size was related to age of fluke and nutrient available to larval stages, flukes in liver were good nourished than those in peritoneum, the ectopic site. Also, fluke extracted from liver fibroses was small in size than those extracted from non-fibroses parenchymatic tissue.

Conclusion

The outcome data, recommended infecting large number of rabbits by 10EMC. This dose protected them from early death and overcome the percentage of failure of infection. Fluke supposed to reach maturity in rabbits 14-16 weeks post infection.

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Explanation of figures

Fig. 1: Changes in ELISA-IgM and IgG in infected rabbits according to EMC dose.

Fig. 2: Pathological changes in liver of rabbits infected by different EMC doses:

A - Liver of rabbit died at 98th d.p.i. after infection by 10 EMC: Liver normal in color, size, with normal gall bladder necrotic foci at site of juvenile fluke penetration (arrow). B - Liver of rabbit infected by 25 EMC died at 74th d.p.i. Enlarged liver with less corrugated surface. Liver capsule thin transparent without thick fibrous adhesion (arrow). C - Liver of rabbit died at 58th d.p.i. (50 EMC/rabbit): Enlarged haemorrhgic rough surface, marked lobule, and fibrins adhesion with surrounding organs. D - Premature *F. gigantica* extracted from liver of rabbits died at 73rd d.p.i.(Carmine stained mounted specimen). E- One *F. gigantica* worm (arrow) detected more superficial protrusion its anterior cone toward the peritoneal cavity from enlarged corrugated liver at 58th d.p.i. (from the group of 50 EMC/rabbit). F - Immature *F.gigantica* worms extracted from liver and peritoneum of rabbits died at 28th & 30th d.p.i. (fresh specimen).