Evaluation of the protective capacity of Toxocara canis embryonated egg antigen in rabbits

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Toxocariasis is a disease of unspecific clinical manifestations in human beings and some animals forming a condition known as visceral larva migrans. Effective Toxocara canis (T. canis) control should destroy the dormant hypobiotic larvae in the tissues of the reservoir hosts. Embryonated egg antigen is intended to be evaluated in rabbits where 15 New Zealand rabbits were divided into 3 groups; immunized group that injected with three doses of T. canis egg antigen (First dose with complete Freund's and two successive doses with incomplete Freund's adjuvant); control infected group and adjuvant control group. At 30th day from the beginning of the experiment, all groups were infected with 10,000 T. canis embryonated egg. Blood samples were collected periodically for measuring antibodies titer by ELISA. Postmortem and histopathological investigations were done. The efficacy of egg antigen protective immunity was evaluated by; the positive antibody titer, retention of larvae in the liver, hepatic eosinophilic granuloma formation, number of the milky spots on the liver surface and lung resistance to the infection. These parameters were detected obviously in the immunized group than the non immunized ones. Consequently, production of this product is recommended where its preparation is simple, easy, fast and economic and may be used as role of vaccine program against the parasite infection in the susceptible hosts.

The effective control of this disease should destroy the dormant hypobiotic larvae in the tissues of the paratenic hosts. Ongoing administration of anthelmintics was neither a practical nor a reliable solution. In contrary, immunization of this host is long lasting and more potent (Barriga, 1988). Immunization of mice with Toxocara larval extracts or metabolic (excretory/secretory) product showed a certain level of immunoprotection and so effective anti T. canis vaccine could be expected in the future (Dvorozankova et al., 2002; El-Zawawy, 2003).

Vaccine efficacy against toxocariasis must be evaluated by; the positive antibody titer, retention of larvae in the liver, hepatic eosinophilic granuloma formation and number of the milky spots on the liver surface and lastly lung resistance to the infection (Sugan and Oshima, 1983; Abo-Shehada and Herbert, 1989; Parsons and Grieve, 1990; Abo–Shehada et al., 1991; Serrano et al., 2001; Cuellar et al., 2001; El-Zawawy, 2003; Frontera et al., 2003).

So, the present work aimed to immunize rabbits using infective stage (egg containing second larval stage) of T. canis and evaluate this vaccine.

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Materials and methods

Preparation of the infective and immunized materials. *Toxocara canis* adult worms were collected from naturally infected puppies of age (2 weeks-2months). The animals were transferred to Parasitology department, Faculty of Veterinary Medicine, Beni-Suef University. These puppies were examined coprologically to show that infected by *T. canis* or not. The infected puppies were treated to expel the worms. These worms were washed by saline then the gravid female worms were incubated in (0.15 M NaCL) at 37°C for 24-72hs to induce eggs laying in which daily collection of worms eggs was occurred, (Barriga and Omar, 1992). Egg embryonation was carried out through its spreading as a single layer in Petri dishes half filled with formal saline, 1% then was incubated at 28°C for 30 days and every 2 days the solution was changed and aerated (Nunes et al., 1997). The infective stage (egg containing second stage larvae, embryonated eggs) were divided into 2 parts, one as infective materials were stored till used and the other part for antigen preparation. The later carried out in which the embryonated eggs were washed several times with 0.01 M PBS (pH 7.4) by centrifugation at 1500 r.p.m. for 10 minutes to remove the formal saline remnant. Then mixing with an equal volume of the same solution was done and homogenized at 6000 rpm for 5 minutes in ice bath. The homogenized sample was sonicated. The suspension was subjected to high speed centrifugation 14,000 rpm for 30 minutes at 4°C. The supernatant containing soluble antigenic materials was separated and stored at -20°C in 1ml plastic vial till used as egg antigen according to (Sabry, 1999). The protein contents of antigenic material were measured using the modified Lowry's Assay, (1951).

Rabbits. Fifteen male of 1.5-2 kg New Zealand rabbits, were divided into 3 groups (five in each) Group (A). Immunized with *T. canis* antigen (immunized *T. canis* group).Group (B). injected with Adjuvant alone as control group (adjuvant control group).Group (C). infected with *T. canis* eggs as control positive *T. canis* group (control infected *T. canis* group).

Experimental design.

Immunization protocol. It was performed according to the protocol described by (Langley and Hillyer, 1989).

In group (A), rabbits were vaccinated against *T. canis* infection by the egg 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 600 µg protein of the prepared antigen (which were obtained from 600 µl of the soluble extract) per animal with Freund's complete adjuvant. The rabbits then injected with 400 µg protein per animal with Freund's incomplete adjuvant at 14 and 21 day from the 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 sites above the shoulder, then the second and third doses were Freund's incomplete adjuvant at 14 and 21 day from the beginning of the experiment respectively. Group C were lifted with out any injection.

Infection protocol. The groups (A, B and C) were infected after 30 days from the beginning of the experiment with 10,000 embryonated *T. canis* eggs (infective stage) orally using stomach tube.

Periods of blood collection and preparation. Blood collected at zero day, 14 and 21 day post immunization and 14, 21, 30, 45 and 60 days post infection (p.i.). The sera were isolated from blood samples and stored till used.

Antibodies detection using Enzyme linked immunosorbant assay (ELISA). ELSA has been developed to detect antibodies titer was carried out according to Moralez et al., (2002). Briefly, ELISA plate was coated with 100 µl/ well of egg antigen at the concentration of 20 µg protein/ml coating buffer after checker board titration which applied to determine the optimal antigen concentration and serum dilution. The plates were incubated overnight at 4°C then washed three times with PBS Tween 20 and blocked with the blocking buffer (200 µl /well), incubated at room temperature for 2h. The plates were washed, 100 µl / well of 1:100 diluted serum samples from infected rabbits and negative sera were added and the plates was incubated for 2 h at 37°C with shaking. Washing occurred and 100 µl / well of anti-rabbit IgG alkaline phosphatase conjugate was added and incubated one hour at 37°C with shaking then were washed. 50 µl / well of substrate solution were added to all wells and the plates were incubated for 30 minutes at 37°C. 50 µl / well of 1 N Na OH were added for stopping the reaction. The optical densities (O. D) were read at 405 nm with a micro-ELISA reader system. The sera were considered to be positive when the absorbance values were as or more than the cut off value (the cut off= double fold of the mean negative sera) according to (Iacona et al., 1980).
Table (1): Mean concentration of antibodies to the different groups using ELISA.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control positive <em>T. canis</em> group</th>
<th>Immunized <em>T. canis</em> group</th>
<th>Adjuvant <em>T. canis</em> group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero day</td>
<td>0.142</td>
<td>0.153</td>
<td>0.139</td>
</tr>
<tr>
<td>14 days post immunization</td>
<td>0.154</td>
<td>0.432</td>
<td>0.225</td>
</tr>
<tr>
<td>21 days post immunization</td>
<td>0.157</td>
<td>0.633</td>
<td>0.252</td>
</tr>
<tr>
<td>15 days p.i.</td>
<td>0.280</td>
<td>0.728</td>
<td>0.375</td>
</tr>
<tr>
<td>21 days p.i.</td>
<td>0.395</td>
<td>0.835</td>
<td>0.413</td>
</tr>
<tr>
<td>30 days p.i.</td>
<td>0.463</td>
<td>0.896</td>
<td>0.478</td>
</tr>
<tr>
<td>45 days p.i.</td>
<td>0.476</td>
<td>0.980</td>
<td>0.479</td>
</tr>
<tr>
<td>60 days p.i.</td>
<td>0.482</td>
<td>0.986</td>
<td>0.484</td>
</tr>
</tbody>
</table>

These readings were the mean and the cut off value (double fold of negative one) was (0.305nm). P.i. = Post-infection

Postmortem and Histopathological pictures.
All rabbits slaughtered at 60 day p.i. The internal organs mainly liver, lung, kidney and spleen were examined macroscopically. For the microscopic examination; small parts were fixed in formalin 10% for at least 24 hours before preparation for histopathological study. Paraffin sections, 5µm thick, were prepared by conventional methods then were stained with (H&E) according to (Bancroft and Stevens, 1996).

Results
Enzyme linked immunosorbant assay (ELISA) antibodies detection. ELISA was performed on sera of different rabbit groups with *T. canis* embryonated egg antigen at zero days from the experiment beginning. 14 and 21 day post immunization then 14, 21, 30, 45 and 60 day post infection (p.i.). The positively threshold value (considered as double fold of mean negative sera) was at O.D (0.305nm). It has been found that in the immunized *T. canis* group; the antibody titer (0.432nm) was found at the 14 days post immunization and reached to (0.633nm) at 21 days post immunization. The titer increased to (0.728nm) at 15 days p.i and then gradually increased to its maximum (0.980nm) and (0.986nm) at 45 and 60 days p.i. Meanwhile, in the infected control *T. canis* group; the first positive mean antibody titer (0.395nm) was at 21 days p.i. and then increased to reach its peak (0.482nm) at 45 and 60 days p.i. Concerning to the adjuvant group, the mean antibody titer (0.375nm) appeared at 14 days p.i. and then increased but did not exceed than (0.484nm) at the end of the experiment (Table 1).

Postmortem findings and histopathological results. The P.M. findings of the immunized *T. canis* group revealed that, the liver surface had large number of small size necrotic foci (milky spots) plate (1, A, A1). Furthermore, the lungs appeared normally, plate (1A, A2). The microscopic picture of liver tissues showed, clear multi eosinophilic encapsulated granulomatus reaction around retained larvae with giant cell formation, plate (2, I, A and B). The most characteristic finding was the retention of the larvae through the liver, plate (2, I, C). Contrary to these, the microscopic picture of the lung appeared normal without granuloma formation, plate (2, I, D).

Concerning to, the infected control and adjuvant groups, the hemorrhagic patches in the lung were obvious, plate (1, B, B2). In addition, the microscopic readings of the lung showed diffuse non encapsulated eosinophilic granuloma, plate (2, II, F). By observing the liver, very few numbers of milky spots were found, plate (1, B, B1). Histopathological findings showed very small number of eosinophilic reaction, plate (2, II, E).

Discussion
Toxocariasis is a frequent helminthiasis that can cause visceral and ocular damage in humans especially in children. Effective *T. canis* control should destroy the dormant hypobiotic larvae in the tissues of the reservoir hosts.

Results of ELISA showed that, the immunized *T. canis* group gave higher titer of immunoglobulins in comparison with the infected control and adjuvant groups, in which the titer increased from the 14th day post immunization and then gradually rose with challenge till the end of the experiment. The immunized material sensitized the immune system as the humeral immune response act to increase the immunoglobulines against *T. canis*.
These results were consistent with that of Parsons et al., (1993); Cuellar et al., (2001); Fan et al., (2003) whom recorded that the T-helper cells played a key role in the capture of migrating larvae of *T. canis* in the liver. In observing ELISA results of the other groups, no immunization so the titer was lower than the immunized group. With respect to the gross pictures of livers in the immunized group, marked numerous small sizes necrotic foci (milky spots) were detected due to the larvae migration and retention in the liver tissues. Added to that, the microscopic pictures showed granulomatous reactions around the retained larvae and did not distribute due to the immunization. This picture was absent in the lung and other tissues. Numerous authors found that the larvae of *T. canis* were trapped in the liver parenchyma post challenge (Sugan and Oshima, 1983; Concepcion and Barriga, 1985; Parsons and Grieve, 1990; Abo –Shehada et al., 1991; Parsons et al., 1993) but they worked on mice not rabbits. It's worthy to clarify that; the immune system ability can not destroy *T. canis* larvae. This opinion strengthens by Badley et al., (1987) who cleared that the release of the surface antigens may be important in allowing larvae to evade the host immune response by facilitating the removal of antibodies and eosinophils from the larval surface. Rockey et al., (1983) found that parasites were able to partially evade interaction with eosinophils in culture by shedding their sheaths. Furthermore, Huer et al., (1989); Lombardi et al., (1990) said that, despite of binding the inflammatory cells however the larvae were able to escape and maintain their infectivity and not damage.

**Plate (1):** A. Macroscopic pictures of immunized *T. canis* group.
A1. Liver surface covered with numerous milky spots due to retained larvae.
A1. Lung appears normal.
**B. Macroscopic pictures of control positive *T. canis* group.**
B1. Liver surface showed few numbers of milky spots due to healing of the lesion post larval migration.
B2. Lung showed hemorrhagic patches.
Plate (2):

I. Microscopic lesions of immunized *Toxocara canis* group.
A. Liver tissue showed granulomatus reaction surrounding remnant of cut Larvae (H&E .stain X 400).
B. Eosinophilic granulomatus tissue in liver (H&E .stain X 200).
C. Granulomatus reaction around retained larvae in liver (H&E .stain X 400).
D. Lung appears normal (H&E .stain X 400).

II. Microscopic lesions of infected *Toxocara canis* group.
E. Liver showed Eosinophilic infiltration due to larval migration (H&E .stain X 400).
F. Lung showed diffuse non capsulated granulomatus reaction (H&E .stain X 400).
El-Zawawy, (2003) reported that *T. canis* immunized mice revealed intense cellular infiltration with abundance of eosinophils and acceleration in the granulomas formation in their liver sections. On contrary to that, the liver of infected control and adjuvant groups, showed minimal granulomatas but the lung of these groups had many granulomatas due to the larvae migrated from liver to lung causing these lesions. On the other hand, the lung appeared free from any spots due to the immunization which prevent the larvae migration to it. This augmented by Serraj et al., (2001) who stated that, the highest degree of the lung resistance was observed in pigs immunized with *A. sum* eggs, while the reduction of white spots following immunization was less evident. Frontera et al., (2003) detected that the immunized pigs developed humeral immune response (IgG) which was negatively correlated to the number of larvae in the lungs, and positively associated with the liver white spots number. The lung of the infected control and adjuvant groups, showed hemorrhagic batches and these due to the migrating larvae and absence of immune response which developed by immunization. The prepared immunized material (larvated eggs) had a great ability for stimulation of the immune responses in the following categories; the retention of the larvae in the liver, elevated antibody titer, a great ability for granulomatus reaction formation and high resistance in the lung against the migrating larvae. Vaccination against the disease was supported by findings of Nicholas et al., (1984) who showed that mice hyper-immunized with *T. canis* (E/S antigen) in adjuvant were significantly resistant to infection. Furthermore, Barriga, (1988) said that hyperimmunization with partially purified extracts of *T. canis* larvae induced (37%) resistance to a challenge in mice administered the extract alone, and it was (76%) when administered with lipopolysaccharide adjuvant. Also, the obtained results came in agreement with Abo–Shehada et al., (1991) who determined that using of ultraviolot irradiated embryonated eggs gave the best protection against reinfection. Besides, Dvorozankova et al., (2002) reported that E/S was more effective in the protection against larval toxocariasis than somatic antigen. Moreover, EL-Zawawy, (2003) showed that immunization of mice with microwaved embryonated eggs of *T. canis* could induce a significant protection against the challenge. So, vaccine production for control of toxocariasis by using egg antigen may be feasible, where its preparation is simple, easy, fast and economic.

In conclusion, the prepared vaccine showed a high degree of the protection through the retention of the larvae in the liver, marked lung resistance, well distinct granulomas formation in liver and elevated antibodies titer. Consequently, production of this vaccine might be recommended since its preparation is simple, easy, fast and economic.

References


the pathology, pathogenesis, and aetiology of disseminated lung lesions in slaughter pigs. APMIS, 111(5):531-538.


