Dept. of Parasitology,
Fac. of Vet. Med., Beni-Suef Univ.

DETERMINATION OF COMMON EPITOPES BETWEEN *TOXOCARA VITULORUM* AND *TOXOCARA CANIS* AS A PRELIMINARY STUDY FOR VACCINE PRODUCTION

(With 2 Tables and One Plate)

By

M.A. EL-ASKALANY; KH.M. EL-DAKHLY and H.S. LOTFY*

* Animal Health Research Institute, Beni-Suef branch.

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تعيين المولدات المضادة المتخصصة والمشتركة بين توكسوكارا الأبقار وتوكسوكارا الكلاب كدراسة مبدئية لإنتاج لقاح ضد المرض

محمود أمين مصطفي العسقلاني ، خالد محمد الداخلي ، حسين سيد لطفي

تمت في هذه الدراسة مقارنة التفاعل المشترك بين توكسوكارا الأبقار وتوكسوكارا الكلاب ، حيث قسم عدد 12 أرنب إلي 4 مجموعات متساوية تم عدوي كل أرنب (في المجموعات الثلاثة الأولي) بعدد 10000 بويضة ناضجة معدية (محتوية علي الطور البرقي الثاني) ، فتم عدوي المجموعة الأولي ببويضات توكسوكارا الأبقار والمجموعة الثانية ببويضات توكسوكارا الكلاب وتم تحصين أرانب المجموعة الثالثة بمولد الضد لبويضات توكسوكارا الأبقار ثم عدواها ببويضات توكسوكارا الكلاب لبيان تأثير العدوى المشتركة ، وأخيرا بقيت المجموعة الرابعة بدون عدوي حيث تم إعطاؤها حامل من نوع فروند. وقد أجري اختبار إنزيم الارتباط المتلازن علي مصل هذه الحيوانات لقياس مستوي الأجسام المضادة ، كما أجري اختبار اللطخ المناعي لبيان الحزم البروتينية المناعية والمسئولة عن إحداث التفاعل المشترك بين نوعي التوكسوكارا. وقد أوضحت نتائج اختبار إنزيم الترابط المتلازن وجود مستوي أجسام مضادة عالي في المجموعة الثالثة والتي تمت فيها دراسة التفاعل المشترك حيث وصلت إلي 0.984 ، 0.958 ، 0.982 ، 0.954 في الأيام 14 ، 21 ، 30 ، 45 بعد العدوى. كما أوضحت نتائج اللطخ المناعي أن الحزم البروتينية بالأوزان الجزيئية 43 ، 40 ، 33 ، 18 والتي ظهرت في الأيام 30 و 45 بعد العدوى مسئولة عن إحداث التفاعل المشترك بين توكسوكارا الأبقار وتوكسوكارا الكلاب.

SUMMARY

In this study, cross reactivity between *Toxocara vitulorum* (*T. vitulorum)* and *Toxocara canis* (*T. canis*) was evaluated. Twelve rabbits were divided into 4 equal groups; the first was infected with embryonated

T. vitulorum eggs (10000 embryonated eggs for each rabbit); the second was infected with the same dose of *T. canis*, the third was immunized with *T. vitulorum* antigen and challenged with *T. canis* and the fourth group given Freund's adjuvant as a control negative group. All these groups were tested by ELISA for detection of serum antibodies. Furthermore, Western Blot was applied to determine the common epitopes between the two species of *Toxocara*. ELISA results revealed that, the mean positive antibody titres recovered from the third group were (0.984), (0.958), (0.982) and (0.954) at the days 14, 21, 30 and 45 p.i respectively. Moreover, immunoblotting revealed that protein bands of molecular weights 43, 40, 33, and 18 kDa respectively at the days 30 and 45 p.i, were common between *Toxocara vitulorum* and *Toxocara canis*. From these bands, prospective vaccine production against toxocariasis could be done.

Key words: *Toxocara vitulorum, Toxocara canis, epitopes, vaccine, rabbits.*

INTRODUCTION

Toxocariasis is considered as one of the important parasitic diseases which constitute a huge public health problem all over the world (Morales *et al.*, 2002). The disease is caused by the nematodal helminth of *Toxocara* spp. including *T. canis* which frequently present in the small intestine of dogs and *T. vitulorum* which inhabits the intestine of buffalo calves (Soulsby, 1982).

Life cycle of *Toxocara* species is so complicated, via which larvae of *T. vitulorum* in white mice for example, manifested a migratory behavior similar to other ascarids. The main route for migration of *T. vitulorum* larvae in buffaloes is from the gut to the liver by passive blood transport through the portal vein and in a minor way, through mesenteric lymph nodes (Roberts *et al.*, 1990). Moreover, migration of *T. canis* larvae through tissues produces pathological changes as well as the larvae became widely disseminated throughout the body, where they could live either encapsulated and/or free in the brain for long periods (Glickman and Summers, 1983). Description of the lesions and migratory patterns of *T. canis* in pigs were studied by (Sommerfelt *et al.*, 2004).

Since the diagnosis of visceral larva migrans is too difficult, immunological methods are required, although little is known about immunity of *T. vitulorum* (Amerasinghe *et al.*, 1984).

The cross reactions were especially prominent between *Toxocara* species, a fact further substantiated when reactivity of *T. canis* E/S-specific monoclonal antibodies were tested against *T. leonina* and *T. vitulorum* antigens. The majority of antibodies precipitated antigens of *T. vitulorum* (Page *et al.*, 1991). Currently, ELISA is being used to detect IgG antibody against *Toxocara* spp. by using the excretory/secretory antigen (E/S) with high sensitivity results, followed by identification of the parasite proteins of this antigen which induced the immune response by the Western blot (Morales *et al.*, 2002). Moreover, Ashour *et al.* (1995) demonstrated the degree of homogeneity and heterogeneity of the ascaridid nematodes with SDS-PAGE protein patterns.

The aim of the present work was to determine the most common epitopes between *Toxocara vitulorum* and *Toxocara canis* that could be applied as a preparatory and feasible step for vaccine production.

MATERIALS and METHODS

1. Collection and embryonation of eggs:

Toxocara vitulorum adult worms were obtained from small intestine of the slaughtered buffalo calves at Beni-Suef abattoirs, washed several times with saline and examined microscopically. Fertile eggs were obtained from gravid females using blunt scissor, sieved, washed and sedimented several times using 1 % formol-saline followed by refrigerating in adequate solution till use (Sabry, 1999). Similarly, sacrificed puppies were eviscerated and dissected, then *Toxocara canis* adult gravid females were collected from small intestine. Fertile eggs were collected, put in clean Petri dishes containing normal saline, followed by washing and finally examined microscopically.

The collected eggs were divided into several amounts put in clean Petri-dishes and incubated at 28 ºC, exposed to several rewashing and good aeration, microscopically examined to observe the developmental changes and left to embryonate for 30 days (Omar and Barriga, 1991 and Mousa *et al.*, 2001). Some of the embryonated eggs were used for antigen preparation, and others were kept at 4 ºC for challenge.

2. Preparation of *Toxocara vitulorum* antigen:

The embryonated infective *T. vitulorum* eggs were washed several times with 0.01 M PBS (pH 7.4) by centrifugation at 1500 rpm

for 10 minutes to remove remnants of the formol-saline. Eggs were mixed with an equal volume of the solution, then homogenized at 6000 rpm for 5 minutes in ice bath. The homogenized samples were sonicated for 5 minutes at 5 pulse rate and 60-80 amplitude value using Cole Parner ultra sonic sonicator. Then the suspension was subjected to high speed centrifugation 14,000 rpm at 4 ºC for 30 minutes. The supernatant fluid containing soluble antigenic materials was separated and stored at -20 ºC in plastic vials till used (Sabry, 1999 and Arafa, 2008). Protein content of the antigenic components was determined according to Lowry *et al.* (1951).

3. Rearing and housing of rabbits:

Twelve male New Zealand healthy rabbits of an average one kg body, reared in clean and disinfected batteries and fed on maintenance ration provided with anti-coccidial drugs. Temperature and ventilation were adjusted.

4. Experimental design:

4.1 Grouping of animals:

Rabbits were divided into 4 equal groups:

- *Group A* (Rabbits infected with *T. vitulorum*).
- *Group B* (Rabbits infected with *T. canis*).
- *Group C* (Rabbits immunized with *T. vitulorum* antigen and challenged with *T. canis*).
- *Group D* (Rabbits administered Freund's adjuvant as a negative control).

4.2. Protocol of immunization:

Rabbits of group (C) were immunized with *T. vitulorum* antigen in Freund's adjuvant according to the method described by Mousa *et al.* (2001).

4.3. Procedure of infection:

Each rabbit was infected with 10000 embryonated *T. vitulorum* or *T. canis* eggs (according to the group) orally using stomach tube.

4.4. Blood collection:

Blood was collected from the ear veins at the days 14, 21, 30 and 45 p.i., then serum was obtained by centrifugation and preserved at -20ºC till use.

5. Diagnostic tools:

5.1. Enzyme-linked immunosorbent assay (ELISA):

ELISA was applied to detect antibodies against *T. vitulorum* antigen using 96-well flat microtiter plate according to Starke-Buzetti *et al.* (2001) and Arafa (2008).

5.2. Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis: (SDS-PAGE):

It was used for determination of molecular weights of polypeptide bands obtained by fractionation of antigen according to Laemmeli (1970).

5.3. Western Blot technique:

Polypeptide bands obtained from SDS-PAGE were electrophoretically transferred to nitrocellulose according Towbin *et al.* (1979) and the results were analyzed using Gel pro-analyzer 3.1.

RESULTS

1- Evaluation of both *T. vitulorum* and *T. canis* infection using ELISA:

ELISA was performed on sera of different rabbit groups (A, B, C and D) with *T. vitulorum* egg larval antigen at **zero** day (at the beginning of the experiment) and at the days 14, 21, 30 and 45 post infection (p.i.). The positively threshold value (considered as double fold of mean negative sera) was at O.D. (0.410). It has been found that, in group (A) the first mean antibody titre was found at the day 21 p.i. (0.496), reached the peak (0.657) at the day 30 p.i and reached (0.566) at the day 45 p.i. In group (B), the first mean antibody titre (0.463) was found at the day 30 p.i. and reached the peak (0.476) at the end of the experiment. Concerning the cross reaction between *T. vitulorum* egg antigen and serum of *T .canis* infected group (group C), there was a marked elevation in the mean antibody titre recording (0.984), (0.958), (0.982) and (0.954) at the days 14, 21, 30 and 45 p.i respectively. In group (D), antibody titres were 0.480, 0.598, 0.778 and 0.743 at the days 14, 21, 30 and 45 p.i. (Table 1).

2- Reaction of *T. vitulorum* antigen with sera of infected (*T. vitulorum* and *T. canis*) groups:

Reaction of *T. vitulorum* antigen with sera of zero day of the experiment not show any bands, while the reaction of *T. vitulorum* antigen with sera of *T. vitulorum* infected group revealed bands of

molecular weights 43, 40, 33, 24, 22 and 18 kDa at the day 30 p.i., the same bands appeared at the day 45 p.i. in addition to bands of 60 and 50 kDa.

Reaction of *T. vitulorum* antigen with sera of *T. canis* infected group revealed polypeptide bands of molecular weights 55, 50, 43, 40, 33, 24 , 22 and 18 kDa at the day 30 p.i., while bands of 50, 43, 40, 33 and 18 kDa appeared at the day 45 p.i.

It's worthy to mention that polypeptide bands of molecular weights 43, 40, 33, and 18 kDa revealed from the reaction of *T. vitulorum* antigen with sera of both *T. vitulorum* and *T .canis* infected groups at both 30 and 45 day p.i.; so these bands were responsible for the cross reaction which was estimated by the ELISA (Table 2 and Plate 1).

Table 1: Evaluation of both *T. vitulorum* and *T. canis* infection using ELISA.

Groups / Time		Group (A)	Group (B)	Group (C)	Group (D)
Zero day		0.206	0.142	0.199	0.208
Day 14 p.i.		0.320	0.284	0.969	0.484
		0.399	0.291	0.993	0.475
		0.309	0.265	0.992	0.481
	Mean	0.343	0.280	0.984	0.480
Day 21 p.i.		0.428	0.394	0.917	0.637
		0.563	0.387	0.984	0.624
		0.497	0.403	0.973	0.532
	Mean	0.496	0.395	0.958	0.598
Day 30 p.i.		0.750	0.454	0.971	0.753
		0.629	0.462	0.985	0.763
		0.592	0.473	0.990	0.818
	Mean	0.657	0.463	0.982	0.778
Day 45 p.i.		0.615	0.472	0.921	0.757
		0.551	0.476	0.980	0.731
		0.531	0.479	0.962	0.742
	Mean	0.566	0.476	0.954	0.743

* Cut off value = 0.410

Table 2: Detection of specific epitopes revealed from reaction of *Toxocara vitulorum* antigen with sera of *Toxocara* species using Western Blot.

Marker	*T. vitulorum* antigen with sera of *T. vitulorum* infected group (day 30 p.i.)	*T. vitulorum* antigen with sera of *T. vitulorum* infected group (day 45 p.i.)	*T .vitulorum* antigen with sera of *T. canis* infected group (day 30 p.i.)	*T .vitulorum* antigen with sera of *T. canis* infected group (day 45 p. i.)
250				
160				
105				
75				
		60		
			55	
50		50	50	50
	43	43	43	43
	40	40	40	40
35				
	33	33	33	33
30				
25				
	24	24	24	
	22	22	22	
	18	18	18	18
15				
10				

Plate 1: The common epitopes revealed from the reaction of *T. vitulorum* egg antigen with sera of both *T. vitulorum* and *T. canis* (1, 1*: Marker)

DISCUSSION

Toxocariasis is one of the most important parasitic problems causing a major public health significance in different areas of the world. The residence of hypobiotic larvae in tissues of infected animals represent the main source of the parasitic persistence in domestic animals owing to migration of these larvae from the tissues of infected mothers to their offsprings via placenta or colostrum, and preceded by pulmonary migration to form adult ascarids in the intestine accompanied by shedding of large number of eggs in the environment.

Concerning to the phenomenon of cross reactivity between *T. vitulorum* antigen and serum of *T. canis* infected group, polypeptide bands of molecular weights 43, 40, 33 and 18 kDa showed cross reaction between *Toxocara vitulorum* and *Toxocara canis* in days 30 and 45 p.i. respectively, and these bands could be detected by using ELISA. These findings were enforced by those obtained by Page *et al.* (1991) who stated that the majority of *T. canis* monoclonal antibodies were precipitated against antigens of *T. vitulorum*.

Similar results were obtained by Safar *et al.* (1992) who estimated that the crude extract of *T. vitulorum* showed cross reaction with *T.canis* and *Ascaris lumbricoides* sera using double gel diffusion. In addition, Ashour *et al.* (1995) showed close relationship between *T. vitulorum*, *Toxascaris leonina*, *Parascaris equorum* and *Toxocara canis*, where protein extracts from adult worms of the four nematodes were resolved into a number of bands. Comparative analysis of dominant bands showed that 13 bands were common among the four species.

Moreover, Magnaval *et al.* (1991) suggested that the high molecular weight fractions were responsible for the cross reactivity. More or less similarity was obtained by Jacob (1995) who observed 4 bands between 29 and 210 kDa. Also these results were in accordance with those found by Sarimehmetoğlu *et al.* (2001) who applied western blot for immunodiagnosis of visceral larva migrans in mice. They revealed that specific protein bands for visceral larva migrans were determined as 24, 28, and 48 kDa.

On the other hand, Iddawela *et al.* (2007) revealed that there was no cross reaction between *Toxocara vitulorum* adult worm antigen and sera of *T. canis* E/S-57 antigen. This might be related to the difference in antigens used.

It is worthy to mention that, a correlation between the band pattern and the stage of infection could be observed; as the antigen-antibody reaction became visible on immunoblotting. Furthermore, a correlation between ELISA results and Western blot must be highlighted. These findings coincided with those revealed by Magnaval *et al.* (1991) and Morales *et al.* (2002).

The heterologus immunity between *Toxocara* species may be helpful for production of common vaccine used for protection against toxocariasis.

REFERENCES

Arafa, W.M. (2008): Some studies on *Toxocara* species in model experimental animal. M.V.Sc Thesis, Fac. Vet. Med. Beni-Suef Univ.

Amerasinghe, P.H.; Massodi, M.A.; Samarasinghe, B.; Sivanathan, S.; Gunawardana, V.K. and Fernando, S.T. (1984): Immunological responses of pregnant swam and Murrah buffalo cows and calves to *Toxocara (Neoascaris) vitulorum* infection. In: The use of nuclear techniques to improve domestic buffalo production in Asia. Int. Atomic Energy Agency, Vienna, Austria., 161–170.

Ashour, A.A.; Taha, H.A. and Mohammad, A.H. (1995): Comparative SDS-page protein patterns of four ascaridid nematodes. J Egypt Soc Parasitol.; 25 (3): 761-767.

Glickman, L.T. and Summers, B. (1983): Experimental *Toxocara canis* infection in cygonomolgus macaques (*Macaca fascicularis*). Am.J.Vet. Res., 44 (12): 2347–2354.

Iddawela, R.D.; Rajapakse, R.P.; Perera, N.A. and Agatsuma, T. (2007): Characterization of a *Toxocara canis* species-specific excretory-secretory antigen (TcES-57) and development of a double sandwich ELISA for diagnosis of visceral larva migrans. Korean J Parasitol., 45 (1): 19-26.

Jacob, C.M. (1995): Ánalise evolutiva dos parâmetros clínico-laboratorais da toxocaríase visceral na infância.São Paulo., (Tese de doutorado–Faculdade de Medicina da USP).

Laemmli, U.K. (1970): Cleavage of structural protein during the assembly of the head bacteriophage T4. Nature., 227: 680-685.

Lowry, O.H.; Rosenbrough, N.J.; Farr, A.L. and Randali, R.J. (1951): Protein measurement with Folin- phenol reagent. J. Biol. Chem., 193: 265-275.

Magnaval, J.F.; Fabre, R.; Maurières, P.; Charlet, J.P. and de Larrard, B. (1991): Application of the western blotting procedure for the immunodiagnosis of human toxocariasis. Parasitol Res., 77(8):697-702.

Morales, O.L.; López, M.C.; Nicholls, R.S. and Agudel, C.O. (2002): Identification of *Toxocara canis* antigens by western blot in experimentally infected rabbits. Rev. Inst. Med .Trop. S. Paulo, 44 (4): 213-216.

Mousa, W.M.; Ezz El-Dien, N.M. and Ibrahim, S.F. (2001): Evaluation of three *Toxocara canis* antigens for serodiagnosis of experimentally infected rabbits with visceral larva migrans. J. Egypt. Vet. Med. Ass., 61 (1): 173–180.

Omar, H.M. and Barriga, O.O. (1991): Biology and pathophysiology of *Toxocara vitulorum* infections in a rabbit model. Vet. Parasitol., 40: 257–266.

Page, A.P.; Richards, D.T.; Lewis, J.W.; Omar, H.M. and Maizels, R.M. (1991): Comparison of isolates and species of *Toxocara* and *Toxascaris* by biosynthetic labelling of somatic and ES proteins from infective larvae. Parasitol., 103 (3): 451-464.

Roberts, J.A.; Fernando, S.T. and Sivanathan, S. (1990): Toxocara vitulorum in the milk of buffalo (*Bubalus bubalis*) cows. Res. Vet. Sci., 49 (3): 289–291.

Sabry, M.A. (1999): Epidemiological studies on Toxocariasis in animals and man. Ph.D. Vet. degree (zoonoses) Fac. Vet. Med., Cairo Univ.

Safar, E.H.; El-Rifaei, F. and Maklad, K.M. (1992): Protein chromatographic study on adult *Ascaris lumbricoides*, *Ascaris vitulorum* and *Toxocara canis*. J. Egypt Soc. Parasitol.,22 (1): 171-176.

Sarimehmetoğlu, H.O.; Burgu, A.; Aycicek, H.; Gönenç, B.; Tanyuksel, M. and Kara, M. (2001): Application of western blotting procedure for the immunodiagnosis of visceral larva migrans in mice by using excretory/secretory antigens. Dtsch Tierarztl Wochenschr., 108 (9): 390-392.

Sommerfelt, I.E.; Rosa, A.; Duchene, A.; Degregorio, O.; Lopez, C.; Pisanu, A. and De Torres, R. (2004): Toxocara canis in experimentally infected pigs. Migratory pattern and tissue lesions. Vet. Parasitol., 125: 323–334.

Soulsby, E.J. (1982): Helminthes, Arthropoda and Protozoa of domesticated animals. 7th ed. Bailliere Tindal & Casell Ltd. London.

Starke-Buzetti, W.A.; Machado, R.Z. and Zocoller-Seno, M.C. (2001): An enzyme-linked immunisorbent assay (ELISA) for detection of antibodies against *Toxocara vitulorum* in water buffaloes. Vet. Parasitol., 97 (1): 55–64.

Towbin, H.; Stachelin, T. and Gordon, J. (1979): Electrophoretic transfer of protein from poly acrylamide gels to nitrocellulose sheets, procedures and some application. Proc. Nati. Acd. Sci. USA., 76:4350-4354.