

DIAGNOSIS OF *TOXOCARA VITULORUM* INFECTION (ASCARIDIDA: NEMATODA): USING SELECTED PURIFIED ANTIGENS

By

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Abstract

Toxocara vitulorum is a nematode parasite of small intestine of buffaloes, particularly young age calves, causing economic and public health problems. Evaluation of the immunogenicity and diagnostic sensitivity of three selected *T. vitulorum* antigens include excretory secretory antigens (ESAg), Pre-enteric Ag (PeAg) and embryonated egg Ag (EEAg) were evaluated on the experimental and field level. PeAg appears as the most sensitive antigen in specific detection of anti-*T. vitulorum* antibodies (ATVAb). In inspected sera using ELISA Fractionation of the three *T. vitulorum* antigens using SDS-PAGE and determination of the specific fractions using EITB versus rabbit-hyper-immune sera (RHIS) and natural infected buffaloes and calve sera revealed that the bands corresponding to M.W of 105, 90, 67, 19 & 11 kDa reacted specifically. Marked specific reaction even with sera from animals infected by low eggs/gram was recorded versus the fraction of 67 kDa in the fractionated PeAg. This work identified this protein fraction as the most specific and sensitive one can be used for routine diagnosis on the field level.

Keywords: *T. vitulorum*, ELISA, SDS-PAGE, EITB, ESAg, PeAg, EEAg.

Introduction

Toxocara vitulorum is a parasite of large ruminants worldwide many tropical and subtropical countries (Rast *et al*, 2014), with a high prevalence in Egyptian cattle and buffaloes calves (Sultan *et al*, 2015). It is responsible for high morbidity and mortality rates resulting in serious economic losses and zoonotic importance (Omar and Lewis, 1993). Humans become infected by ingestion of infective eggs either from soil, dirty hands, raw fruits and vegetables or larvae from undercooked meat and unpasteurized milk (Borecka *et al*, 2010). Migration of the larvae through tissues produces severe inflammatory reactions and consequently to a wide range of pathological and clinical manifestations (Strube *et al*, 2013). Coprological examination techniques fail to detect infection in adult buffaloes and also during the prepatent stage and in mild infections. So in case of absence of parasitological evidence of infection, the immunological methods considered to be the only available way for diagnosis of infection (Jyoti *et al*, 2011). Serological tests based on immunological techniques Such as ELISA were recognized

as the most effective approach for laboratory diagnosis of toxocariasis, (Fillaux and Magnaval (2013). Also, SDS-PAGE and western blotting considered more beneficial in identification of specific *Toxocara* spp. diagnostic or immunogenic protein fractions (Pinelli *et al*, 2013). Several *Toxocara* antigens were extracted by many authors and diagnostic values were determined by ESAg (Starke-Buzetti and Ferreira (2006), EEAg (El-Askalany *et al*, 2008), and PeAg (Jyoti *et al*, 2011)

The present study aimed to evaluate three *Toxocara vitulorum* antigens (ESAg, PeAg & EEAg) in diagnosis of *T. vitulorum* on the suspected level and known infected ones. Fractionation of antigens was performed using SDS-PAGE followed by EITB techniques to determine the most specific diagnostic kDa antigenic fractions on field level to diagnose zoonotic visceral larva migrans.

Material and Methods

All the steps and procedures were approved by the Institutional Animal Care and Use ethical Committee (CU-IACUC), Cairo University (CU/ II/ S/ 2016/ 2017).

T. vitulorum antigens: Intact, active *T. vit-*

ulorum adult worms were collected from intestine of freshly slaughtered buffalo calves in Cairo Governmental Abattoir. Worms were well washed in normal saline solution to prepare ESAg and peAg. *T. vitulorum* eggs were collected from the uteri distal part of adult females then embryonated for preparation of EEAg and induction of infection of rats (Starke and Ferreira, 2006).

T. vitulorum excretory/ secretory antigen (ESAg): ESAg was collected after incubation of active adult worms at 37°C for 2 hrs in PBS supplemented with antibiotic. The fluid was centrifuged at 460xg for 5 minutes and the supernatant was filtered (Amerasinghe *et al*, 1992). The protein content was increased by concentration using polyethylene glycol in molecular porous membrane tubing 6-8 MW cut off (Spectrum Medical Inc., Los Angeles, CA 900060) at 4°C (Goswami *et al*, 2013). The concentrated fluid was collected and its protein content was measured using Lowry's Assay (1951) then stored at -20°C till use.

T. vitulorum perienteric fluid antigen (PeAg): PeAg was collected from adult active *T. vitulorum* by puncturing the posterior end of each parasite with needle and perienteric fluid was drained, collected and centrifuged at 460xg for 5 minutes then treated as ESAg (Ferreira and Starke-Buzetti, 2005).

Embryonated eggs antigen (EEAg): EEAg was prepared from the previous embryonated *T. vitulorum* eggs after several washing with 0.01 PBS (PH 7.4) by centrifugation at 1500 r.p.m. for 3 minutes. Sediment eggs were mixed with an equal volume of the same solution then homogenized at 6000 r.p.m for 5 minutes in an ice bath. Homogenized sample was sonicated for 5 minutes at 5 pulse rate and 60-80 amplitude value using Coleparmer Ultrasonic homogenizer then suspension was subjected to high speed centrifugation at 14,000 rpm for 30 minutes at 4°C and the supernatant was separated as EEAg, concentrated and its protein contents was measured and stored (El-Askaalany *et al*, 2008).

Production of hyper-immune sera in rabbits (RHIS): The method adopted was that of Tang *et al*. (2015), with little modification. The prepared *T. vitulorum* antigens (ESAg, PeAg & EEAg) were used to produce hyperimmune sera. Sera were collected before immunization as negative sera. Three rabbits for each antigens were immunized subcutaneously with 1.2 mg protein of *T. vitulorum* antigens, emulsified 1:1 with mineral oil. After 2 weeks another 1.2 mg protein was mixed 1:1 in the oil and divided into 3 doses (day 14, 21 & 28) then injected subcutaneously. One week after the last immunization; rats were bled for sera and antibodies level was measured, and stored at -20°C until used as positive sera.

Tested sera: Fifteen natural infected buffaloes and 15 natural infected buffaloes-calves sera from three different levels of eggs/gram as well as 10 non-infected buffaloes and calve sera were used to evaluate antigens.

ELISA: Assay was done after Liu *et al*. (2015) with little modification. Optimal dilutions of various reagents were determined using Checkerboard titration. Microtitration polystyrene plates, 96-well, was coated with 4µg protein/ml from each of the 3 tested antigen in coating buffer (200µl/well), incubated overnight at 4°C. Plates were washed three times with washing buffer (0.05% Tween-20 in PBS pH 7.3) then blocked using 0.5% bovine serum albumin in PBS-Tween-20 (200µl/well) and incubated at 37°C for an hour. After washing reagents (100µl/well) were added and plates incubated at 37°C for an hour with shaking and washing after each step: tested and control serum samples (1:100 dilution). Protein A horse reddish peroxidase conjugate (1:1000 in PBS) was detected by adding 100µl/ well of substrate o-phenylenediamine dihydrochloride (OPD), plus H₂O₂ for 30 minutes and stopped by adding 50µl/well of 1N H₂SO₄. Optical densities (O.D) were read at 450nm with a micro-ELISA reader system. Sera were considered positive when absorbance values were as or more than the cut off value

(cut off= double fold of mean negative control sera). Test evaluated diagnostic efficacy of antigens versus positive and negative RHIS as well as suspected field collected samples. Fractionation of antigens & transferring of protein: SDS-PAGE analysis of 3 tested antigens under reduced conditions was done using 12% non-gradient slab gel with stacking gel 5%. Samples were electrophoresed at 20 mA current (Laemmli *et al*, 1970). Gel was shifted to coomassie blue stain and calibrated with marker in range of 230-10 KDa. Gel proteins were transferred at 10V, 100 mA overnight at 4°C, onto nitrocellulose paper (Towbin *et al*, 1979). Sheet was dried and stored until use.

Determination of specific protein fractions using EITB: Each *T. vitulorum* antigen was tested versus own RHIS to detect specific protein fraction. Also antigens were tested versus positive sera of buffaloes showed no eggs in feces and infected buffaloes and buffalo calves had eggs in feces to detect specific band to diagnose field infection. A longitudinal NC strips (15x0.5cm) with the fractionated antigens were cut out. Non-specific binding of antibodies was blocked by incubating the strips with 3% BSA TNT at 37°C for 1 h. After blocking, strips were incubated versus known positive and negative control sera at 1:100 dilution, 0.5ml of sera/strip for 2 hours using Western-blot

assay (Towbin *et al*, 1979). After washing, NC strips were exposed to 1:1,000 HRP-conjugated IgG (Sigma, USA), diluted in blocking buffer, at 37°C for 1 h. Peroxidase substrate (4-chloro-1-naphthol) was added to reveal IgG/ antigen interaction. Protein fractions positively reacted with positive control sera, and negatively with negative control one as specific protein fractions.

Results

All *T. vitulorum* antigens were sensitive for ATv-Ab in the infected animals' sera. PeAg was the most sensitive one produced highest O.D values with increasing of sera dilution upto 1:200 in comparison with the other 2 antigens. The fractionation of tested antigens using SDS-PAGE revealed bands in the range of (187-17; 165-15 and 135-28) kDa for ESAg, PeAg and EEAg respectively.

The ESAg gave seven bands corresponding to M.W standard at the level of 187, 115, 80, 67, 43, 30 & 17 kDa. Eight protein bands were detected in fractionated PeAg correlated to M.W standard at the level of 165, 126, 111, 67 KDa, 54, 45, 20, & 15 kDa. F

The fractionation of EEAg revealed six bands corresponding to M.W standard at the level of 135, 105, 80, 67, 43 & 28 kDa.

Statistical analysis: Data were tabulated and analyzed.

Details are shown in tables (1, 2, & 3) and figures (1, 2 & 3)

Table 1: Diagnostic value of tested *T. vitulorum* antigens versus known infected sera.

EPG of the inspected animals' feces		Serum dilution	Mean ELISA O.D versus		
			ESAg	PeAg	EEAg
Infected buffaloes (n= 5 animal/group)	<300	1: 100	0.396	0.482	0.333
		1:200	0.311	0.441	0.302
	≥300-500	1:100	0.511	0.593	0.442
		1:200	0.438	0.522	0.408
	>500	1:100	0.565	0.683	0.501
		1:200	0.488	0.579	0.416
Infected buffalo calves (n= 5 animal/group)	<1000	1:100	0.655	0.893	0.522
		1:200	0.529	0.799	0.418
	≥ 1000-2000	1:100	0.712	1.012	0.596
		1:200	0.603	0.867	0.525
	>2000	1:100	0.748	1.133	0.623
		1:200	0.611	0.977	0.558
Control (n= 5 animal/group)	Buffaloes	1: 100	0.110	0.103	0.106
		1: 200	0.093	0.089	0.091
	Buffalo calves	1:100	0.091	0.096	0.102
		1: 200	0.083	0.076	0.087

Table 2: Protein fractions characteristic to different *T. vitulorum* antigens using Commassie stain SDS-PAGE

MW Marker	ESAg	peAg	EEAg
230	187	165	135
150	115	126	105
100	80	111	80
80	67	67	67
60	43	54	43
50	30	45	28
30	17	20	
25		15	
20			

Table 3: *T. vitulorum* antigenic fractions reacted specifically versus positive and negative RHIS.

Marker	ESAg versus		PeAg versus		EEAg versus	
	+ve RHIS	-ve sera	+ve RHIS	-ve sera	+ve RHIS	-ve sera
230	105	150	126		135	150
150	80		105		88	25
100	67		74		67	
80	50		67		28	
60	45		31			
50	43		11			
40	30					

Testing of the antigens on NC strips versus its own RHIS revealed seven bands of M. W 105, 80, 67, 50, 45, 43 & 30 kDa, six bands at M.W of 126, 105, 74, 67, 31 and 11 kDa and four bands at M.W of 135, 88, 67 & 28 kDa for *T. vitulorum* ESAg, PeAg and EEAg respectively. It was noticed that the protein band of M.W. 67 kDa was found to be specific for *T. vitulorum*. Moreover, testing of the *T. vitulorum* antigens versus control negative serum, some showed reaction toward other non-specific fractions (150 KDa & 25 KDa). While after the previous EITB reaction was repeated using sera of *T. vitulorum* infected buffaloes with low and high EPG, sera from previously ELISA diagnosed buffaloes showing no eggs in feces, buffalo calves showing high EPG in feces as well as negative control sera, it was revealed that there was marked reaction of the fractionated antigens towards bands of M.W 105, 90, 67, 19 & 11 kDa which didn't cross react with the other control sera. It was important to mention that the fraction of 67 kDa from the fractionated PeAg reflex the more prominent reaction.

Discussion

Gastrointestinal (GI) parasitism is a major constraint for livestock production causing heavy economic losses to the livestock producers. It plays a crucial role in reducing

animal production by lowering the working capacity, growth, body weight and milk yield (Choubisa and Jaroli, 2013). Sometimes, diagnosis *T. vitulorum* was not easy as the larvae migrate in the tissues, remaining as dormant or hypobiotic parasites, transmitted through colostrum and milk, causing disease (severe anemia, diarrhea, weight loss & anorexia) particularly in buffalo calves (Wickramasinghe *et al*, 2009).

In the present study, as to evaluation of these antigens on field level versus sera of natural infected buffaloes and their calves, it was found that all *T. vitulorum* antigens were sensitive for detection of (ATv-Ab) in the infected animals' sera but the PeAg appeared as the most sensitive one as it produced the highest O.D values in comparison with the other two antigens. These results agreed with Souza *et al*. (2004) who reported that the *T. vitulorum* PeAg was more sensitive and specific than the ESAg in the term of detection of highest levels of antibodies in sera of infected buffaloes and buffalo calves. Also, Ferreira and Starke-Buzetti (2005) in Brazil reported that *T. vitulorum* PeAg was specific and sensitive for detection of (ATv-Ab) in infected buffaloes and buffalo calves sera. Moreover, Thomas *et al*. (2016) who used ESAg and EEAg in diagnosis of toxocariasis in India reported that

these antigens lack sensitivity and specificity to detect infection and showed a high cross reaction with other Ascarid antigens.

In the present study, electrophoretic analysis of the tested *T. vitulorum* antigens revealed 7, 8 & 6 bands ranged from (187-17), (165-15) and (135-28) kDa for ESAg, PeAg and EEAg respectively. These results agreed with Starke-Buzetti and Ferreira (2006) they reported that fractionation of *T. vitulorum* ESAg revealed 8 bands in range of 190-19 kDa. While Jyoti *et al.* (2011) who performed in India an electrophoretic analysis of *T. vitulorum* PeAg that showed 7 bands of MW 20.3, 43.6, 53.1, 57.4, 66.9, 111.3 and 126.3 kDa. Moreover, Ferreira and Starke-Buzetti (2005) reported that *T. vitulorum* PeAg was fractionated into 9 bands that ranged from 165- 11 kDa.

The variations in the M.W. may refer to the method of antigen preparation, type of the used gel, time and voltage adjusted within the SDS-PAGE. Each *T. vitulorum* antigen was tested versus its own RHIS to detect the specific protein fraction of each one. Also, Pinelli *et al.* (2013) stated that western blotting considered more beneficial in identification of specific *Toxocara* spp. diagnostic or immunogenic protein fractions. The reaction revealed seven bands of M.W 105, 80, 67, 50, 45, 43& 30kDa, six bands at M.W of 126, 105, 74, 67, 31and 11 kDa and four bands at M.W of 135, 88, 67& 28 kDa for *T. vitulorum* ESAg, PeAg and EEAg respectively. Also it was cleared that band of M.W 67 kDa was found to be specific band for *T. vitulorum*. This result agreed with Jyoti *et al.* (2011) in India who reported that band of 66.9 kDa was found a specific for *T. vitulorum*. It was important to detect the specific band that can be applicable in diagnosis of *T. vitulorum* infection at field level to avoid the non-specificity and cross reactivity problem. Thus, testing of each *T. vitulorum* antigen versus positive sera of buffaloes and buffalo calves was done. There was marked reaction of the fractionated antigens towards bands of M.W 105, 90, 67, 19 & 11

kDa. Also, Jyoti *et al.* (2011) recorded in India bands of M.W 105, 66.9 and 11 kDa in highly infected buffalo calves sera. But, Ferreira and Starke-Buzetti (2005) detected bands of M.W 165, 112, 88, 76, 31 & 11 kDa by testing PeAg versus positive calve sera with high and low EPG. Fillaux and Magnaval (2013) reported bands of M.W 190, 110, 90, 64 & 11 kDa by using ESAg.

Conclusion

The 67 kDa from the fractionated PeAg reflex was more prominent reaction with sera of naturally infected animals. This band proved marked sensitivity to detect ATv-Ab even in positive samples of low ELISA/OD and low EPG. This band extracted from PeAg is specific to diagnose toxocariasis in epidemiological survey and laboratory.

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Explanation of Figures

Fig. 1: Commassie blue stained SDS-PAGE gel containing fractions of different *T. vitulorum* antigens.

Fig. 2: Protein fractions of *T. vitulorum* antigens on NC membrane treated versus positive and negative RHIS.

Fig. 3: Protein fractions of *T. vitulorum* antigens on NC membrane treated versus positive and negative buffalo and buffalo calves sera

