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Detection of Antibodies to Excretory-Secretory Antigen of *Toxocara vitulorum* Infective Larvae in Buffalo Calves by ELISA

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Abstract: In the current research, -immunogenic binding activities of *Toxocara vitulorum* second larvae somatic antigen and excretory secretory antigen (TvL_2ESA) were studied by ELISA using hyperimmune serum. As, the excretory secretory antigen was the most potent one. So, it was selected for diagnosis of toxocariasis in buffalo calves, whereas it recorded infection percentages of 73.8. Structural characterization of TvL_2ESA was done by SDS-PAGE and it revealed 8 bands of molecular weights ranged from 5 - 135 KDa. Further characterization of this antigen was carried out by immunoblot assay in which naturally infected buffalo calves sera were utilized. Only three bands of molecular weights 71, 51, 24 KDa were identified. Free amino acid analysis of the same antigen revealed 18 amino acids with high proportion of only two of tyrosine and aspartic. In conclusion: ES products of *T. vitulorum* second larvae was the most diagnostic antigen and was successful in the detection of high infection percentage of toxocariasis among buffalo calves in Egypt.

Key words: Toxocariasis · Immunodiagnosis · Immunoblot · SDS-PAGE

INTRODUCTION

Toxocara vitulorum is an important pathogene in the small intestine of buffaloes, cattle and calves. It is also widly spread in tropical countries and causes much morbidity and mortality among calves [1].

A diagnosis of this parasite could be carried out by detecting eggs in faeces. However, fecal egg counts may give false negative results due to infection with immature worms or mature worms of one sex [2] or false positive results due to coprophagia [3]. Alternative immunodiagnostic approach would represent a considerable advantage for clinical and epidemiological studies. In this respect, the identification of specific antigen that can induce potency in the diagnosis is crucial [4]. Immunodiagnosis of toxocariasis was previously probed utilizing different extracts. Antibodies against larval excretory secretory product (ES) of *T. vitulorum* were detected in serum of naturally infected buffalo calves by ELISA [5]. In addition, anti *T.vitulorum* antibodies were detected in the colostral samples from infected buffaloes by ELISA using somatic and excretory secretory antigens of second larval stage [6]. It was proved that ES antigen is the most potent antigen. Also, the ES antigen of *T. canis* larvae has been considered as a major functional antigen in immunodiagnosis of toxocariasis [7]. ELISA using soluble antigen of *T.vitulorum* infective larvae was successfully used to understand the development of immune response in infected calves [8,9]. While, 5 *T. vitulorum* antigens (adult, eggs, ES for males and females worms and coproantigen) were used in the diagnosis of naturally toxocariasis in buffalo calves by ELISA [10]. They declared that adult antigen is the most potent extract. Furthermore, -De Souza *et al.* [11] showed by ELISA that the highest level of antibodies against perienteric antigen (Pe) of *T. vitulorum* were detected in buffalo cow sera during the prinatal period and were maintained in high level through 300 days after parturition. Ghosh and Banerjee [12] evaluated 4 diagnostic assays for diagnosis of toxocariasis in buffaloes using larval somatic and excretory secretory antigens. The evaluation showed ELISA as the most sensitive technique whereas, it detected antibodies as early as seven days post infection. Furthermore, ELISA based on protein fraction of 57 KDa of *T. canis* ES antigen was proven to be an effective tool in the diagnosis of human toxocariasis [13].

The purpose of this study was to compare the immunogenic binding activities of *T. vitulorum* second larvae somatic and excretory secretory antigens. Also, the most potent antigen to be used in the diagnosis of toxocariasis by ELISA was selected. Moreover,

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characterization of the most immunodiagnostic antigen by SDS-PAGE, immunoblot assay using sera from *T. vitulorum* naturally infected buffalo calves and analysis of antigen's free amino acids was done.

MATERIALS AND METHODS

Serum Samples: A total number of 88 serum samples were collected from buffalo calves slaughtered at Cairo abattoir during the period from June 2008 to April 2009. Samples were labeled and stored at -20°C until use for serological diagnosis.

Rabbit Hyperimmune Serum: Rabbit anti *Toxocara vitulorum* antiserum was raised according to Fagbemi *et al.* [14] with slight modifications. The protocol started by a single subcutaneous injection of 40 µg/kg of the antigen emulsified in complete Freund's adjuvant followed by a single subcutaneous injection of 40 µg/kg antigen in incomplete Freund's adjuvant after two weeks from the first one. Second and third booster doses were given on days 21 and 28, respectively without adjuvant. Blood samples were collected 4 days after the last immunization from ear vein. Collected antiserum was heated at 56°C for 30 minutes for decomplementation, aliquoted and stored at -20°C until use.

Preparation of TvL_2ESA: Eggs were collected from mature female worms and incubated in 1% formaline at 28°C for 17-21 days until most of them were contained larvae [15]. The samples are aerated and solution was changes every 3 days. Embryonated *T. vitulorum* eggs were artificially hatched after decortications according to procedures of Croweroft and Gillespie [16]. Sediment from embryonated eggs was collected after washing several times in PBS, then combined with an equal volume of sodium hypochlorite solution (14% available chlorine) and incubated for 20 minutes at room temperature until the eggs were completely decorticated. At least 10 washes in PBS were carried out. The decoated egg were suspended in PBS and placed in a water bath at 37°C for 1h while air was bubbled with a Pasteur pipette through the suspension until the larvae were hatched. The larval suspension was recovered by centrifugation and the sediment was suspended in PBS for 3 h, The ES product was collected by centrifugation at 10000 rpm for 30 minutes, the spernatant were collected, concentrated and examined for protein content by the method of Lowry *et al.* [17]. Aliquoted and stored at -20°C until use

Preparation of Larval Somatic Antigen: After eggs hatching as described above, the hatched infective larvae were collected using Baermann's apparatus according to the method described by De Savigny [18].The collected larvae were homogenized in 0.15M PBS pH 7.2 using Teflon glass homogenizer followed by sonication.The homogenate was centrifuged at 15000 rpm for 45 minutes at 4°C. The protein content of the supernatant was determined using the method of Lowry *et al.* [17]. The antigen was aliquoted and stored at – 20°C until use.

Enzyme Linked Immunosorbent Assay (ELISA): The antigenic activities of both *T. vitulorum* second larval stage somatic and excretory secretory antigens against two fold serially diluted hyperimmune serum were monitored by ELISA. The assay was also adopted to diagnose *T. vitulorum* infection among buffalo calves using TvL_2ES antigen. Dilution of buffalo calves sera was determined by checkerboard titration together with antigen concentration and horse radish peroxidase dilution. ELISA was performed according to Santiago *et al.* [19] with slight modification. The cut off point for the OD values was determined as described by Murrell *et al.* [20].

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): The gel cast comprised 10% separating and 2% stacking gels and applied to 10 µg protein/well. Mini-protein 11 Dual slab cell (Bio-Rad-Labs, Richmond; CA) was used to conduct electrophoresis using discontinuous system of Laemmli [21]. The TvL_2ES antigen was diluted in sample buffer under reducing condition. The gel was stained with 2.5% Coomassie blue R-250 in fixative (45% methanol-5% acetic acid and 50% distilled water). Destained for over night. Analysis of the separated bands was performed by software analysis Gel Pro Analyzer 3%.

Immunoblotting: The antigen was separated by SDS-PAGE and then electrically transferred onto nitrocellulose membrane at 100v for 1h at room temp in running buffer [22]. Nitrocellulose sheets were cut into 0.5 cm strips followed by blocking in 5% BSA in PBS for 2 h on rocker platform. Diluted naturally infected sera at 1:100 in 5% BSA/PBS Tween were reacted with fractionated TvL_2ES antigen on nitrocellulose strips for 2h on a rocker platform. After washing, the strips were incubated with alkaline phosphatase labeled antibovine IgG (Sigma) diluted at 1:3000 in PBS-T for 1h on a rocker platform. Then exposed to substrate solution (BCIP/NBT

tab; Sigma Co.,) for 10 min. Nitro cellulose sheets were rinsed with distilled water to stop the reaction. The separated bands were analysed by Gel Pro analyzer 3.1.

Amino Acid Analysis: Amino acid composition was determined after hydrolysis in HCl prior analysis with amino acid analyzer (GBC Australia).

RESULTS

Evaluation of Immunodiagnostic Potency of TvL_2ES and Somatic Antigens: The antigenic activities of the two antigens were evaluated by ELISA in which hyperimmune serum was utilized. As shown in Fig. 1, ES antigen was the most potent one. So it was selected for diagnosis of toxocariasis in naturally infected buffalo calves.

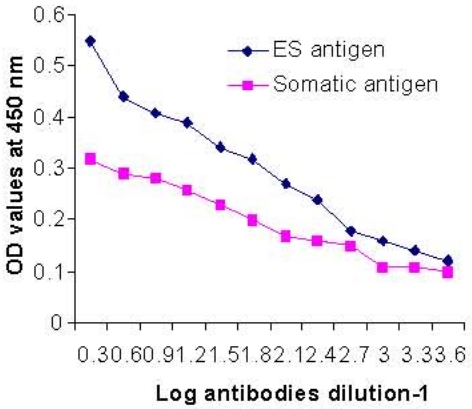

Fig. 1: Comparative evaluation of the immunogenic binding activities of *T.vitulorum* somatic and ES antigens

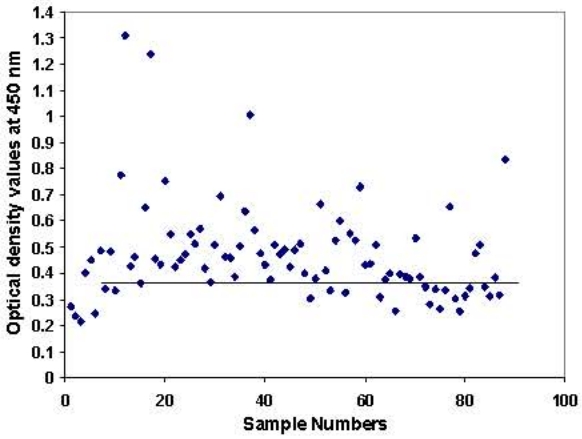

Fig. 2: Scatter graph represents the potency of TvL2ES antigen in the diagnosis of toxocariasis among buffalo calves (Horizontal line represents cut off value)

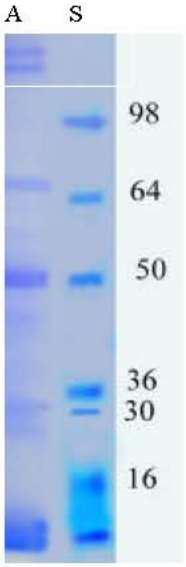

Fig. 3: SDS-PAGE of TvL_2ES antigen, Lane S: Molecular weight standards. Lane A: TvL_2ES antigen

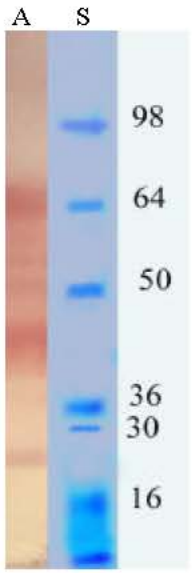

Fig. 4: Immunogenic bands of TvL_2ES antigen identified in immunoblot assay using sera from naturally infected buffalo calves. Lane S: Molecular weight standards. Lane A: TvL_2ES antigen

Diagnosis of Toxocariasis: Detection of antibodis against *T. vitulorum* infection was performed in buffalo calves using TvL_2ES antigen. The assay proved that 65 out of 88 random serum samples were positive for *Toxocara* infection recording 73.8 infection percentage. As indicated by the obtained OD values, the positive samples showed different degree of positivity as depicted in Fig. 2.

Table1: Analysis of free amino acids of TvL_2ES antigen

Amino Acid	Content (mg/g)	Amino Acid	Content (mg/g)	Amino Acid	Content (mg/g)
Asparatic	4.741	Arginine	1.59	Methionine	0.39
Glutamic	2.354	Therionine	0.51	Cystein	1.45
Serine	1.745	Alanine	1.61	Isoleucine	0.23
Glycine	1.359	Praline	0.82	Leucine	0.86
Glutamine	1.44	Tyrosine	8.23	Phenylalanine	1.57
Histadine	2.67	Valine	0.41	Lysine	1.85

SDS-PAGE: The electrophoretic profile of TvL_2ES antigen is shown in Fig. 3. The antigen was resolved into 8 bands of molecular weights 135, 122, 71, 51, 33, 24, 8 and 5 KDa.

Immunoblot Assay: Three immunogenic components were detected in TvL_2ES antigen using calves naturally infected sera (Fig. 4) with molecular weights of 71, 51 and 24 KDa.

Amino Acid Analysis: For additional structural characterization of TvL_2ES antigen, analysis of its free amino acids was undertaken. The antigen exihibited 18 amino acids as shown in Table 1. Notably, the antigen was rich in tyrosine (8.23) and aspartic (4.74), while, isoleucine (0.23), methionine (0.39), valine (0.41) and therionine (0.51) were present in minute amounts

DISCUSSION

The coprological diagnosis of *T. vitulorum* infection is not so accurate [2,3]. Because the positive diagnosis by this method requires mature worms producing eggs, extensive hepatopulmonary larval migration with resulted pathologic changes have occured before such diagnosis is possible. Early diagnosis via immunological techniques would allow initiation of therapy before extensive larval migration and resultant pathologic changes. Accuracy of immunodiagnosis depends on the development of satisfactory extract and successful choice of the assay. The selection of ELISA in the present research was based on previous studies suggesting ELISA as good tool for serodiagnosis of toxocariasis [12, 13, 23].

In the current research, TvL_2ES antigen showed the most potent activities compared with somatic antigen by ELISA and this coincided with Ghosh *et al.* [6], and De Souza *et al.* [11] whereas, they compared between *T. vitulorum* larval somatic and ES antigen using ELISA. They proved that ES antigen was the most potent in imunodiagnosis of toxocariasis. Also, ES antigen of *T. canis* infective larvae has been considered as a major functional antigen in immune response against toxocariasis [7].

The characterization of parasite antigens is essential step for further understanding host-parasite relation ships and is particularly important in the case of human tissue-invasive parasites. In this investigation, the larval excretory-secretory antigen was analyzed and it had been shown that it may be of paractical value in diagnosis of toxocariasis. Analysis of TvL_2ES antigen was adopted by one dimensional SDS-PAGE and showed eight bands in both high and low molecular weight ranges (5 - 135 KDa). The same number of bands but with different molecular weights was detected in ES of *T. vitulorum* larval antigen [24, 25]. This difference in the molecular weights may be due to the different methods used in the antigen preparation. In addition, one band, 51 KDa, of those observed in the current research has molecular weight comparable to band of 50 KDa which associated with adult worm of *T. vitulorum* [26].The antigens of infective larvae, particularly larval ES, were highly effective immunogens. Four injections of ES antigen with no adjuvant induced >92% protection. Even single injection of ES antigen induced significant protection [1].

In the current research three reactive components of molecular weights 71, 51and 24 KDa were detected by immunoblot assay using calves naturally infected sera. In previous studies, four immune reactive bands with high molecular weights of 190, 150, 110 and 90 KDa were recognized in TvL_2ES antigen using sera and colostrum of buffaloes naturally infected with *T. vitulorum* [24]. In addition, one immunogenic band of molecular weight 24 KDa which detected in the present study was previously detected by Mousa *et al.* [25] in *T. vitulorum* larval antigen during the course of immunization and post infection and it is considered as one from three protective bands.

To add further information on the structure of ES antigen its free amino acid composition was analyzed. It was concluded that the ES antigen is consisted of 18 amino acids with high proportions of only two of them tyrosin (8.23) and aspartic (4.74). To our knowledge, no data are available about amino acids analysis of TvL_2ES antigen, but tyrosine was previously detected with high

amount in pure fraction of *T. vitulorum* adult antigen [26] and pure fraction of *Trichinella spiralis* larval extract [27]. Furthermore, Cox *et al.* [28] observed high proportion of glutamic acid and asparagin in *Haemonchus contortus* third larval stage. They not detected tyrosine which is presented in high amount in the present study, although both worms are nematode. Timanova *et al*, [29] analyzed the N-terminal amino acid sequence, of a 12KDa fatty acid binding protein of *A. suum* reproductive tissues and identified N-terminal 33 amino acid residues.

From these previous studies together with the present one, it could be conclude that, each helminth has its own amino acid structure and unique proportion of each acid. It is of great importance, therefore, to identify the amino acid composition of the TvL_2ES antigen particularly when it proved potency in serodiagnosis. Because this will add to the structural details of the antigen as an essential step for its synthetic production which must be preceded with determination of amino acid sequence that could be undertaken in further investigations.

It could be concluded that ES antigen of *T. vitulorum* second larvae stage showed marked potency in the diagnosis of toxocariasis. Consequently, the use of this antigen might be of great value in the control and epidemiological studies of this important and economic parasite. Further studies are necessary for obtaining high purified antigen with high satisfactory results.

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