Characterization of excretory-secretory antigens of adult *Toxocara canis* by western blotting

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Received: 2 October 2012 / Accepted: 11 November 2012 / Published online: 29 November 2012
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Abstract *Toxocara canis* is one of the most common helminth worm of dogs which continues to stimulate both public health concern alongside the higher scientific interest. It may cause visceral and ocular damage in humans especially in children. The identification of specific antigens of *T. canis* is important so as to develop better diagnostic techniques. Excretory-secretory (ES) antigens were prepared by culturing the adult *T. canis* worms in RPMI 1640 medium without serum supplementation followed by ammonium sulphate precipitation. These antigens were separated using sodium dodecyl sulphate-electrophoresis (SDS-PAGE). Recovered proteins ranged from 30 to 384 kDa. The specific reactivity of the *T. canis* excretory-secretory (TC-ES) proteins was checked by western blotting. The immuno-reactivity of the naturally infected dog sera with the TC-ES antigens showed five bands at 43, 57, 105, 139 and 175 kDa. The immuno-reactivity of the hyper immune serum raised in rabbits against TC-ES antigens was observed with ten polypeptides of 21, 25, 30, 37, 45, 50, 57, 69, 77 and 105 kDa. Common antigens band were observed at 57 and 105 KDa. These antigens merit further evaluation as candidate for use in diagnosis of toxocariasis in humans and adult dogs.

Keywords Diagnosis · ES-antigens · *Toxocara canis*

Introduction

Toxocara canis is a wide spread zoonotic gastrointestinal nematode parasite of dogs and other canids (Glickman and Schantz 1981; Lewis and Maizels 1993; Despommier 2003). The more classical zoonotic emerging diseases like toxocariasis are dealt with increased scientific interest (World Health Organization 2004). It is a disease of non specific clinical manifestations and is characterized by visceral larval migration in humans and certain animals. In the absence of parasitological evidence of infection particularly in humans, immunological methods are better requisite for its diagnosis (Glickman and Schantz 1981). Characterization of proteins that comprise adult *T. canis* worms may prove useful in the development of vaccine, diagnosis of infection and in studies on the biology and immunity of the parasite. The excretory-secretory proteins and associated carbohydrates of the third stage larvae of *T. canis* are important candidates in this regard (Maizels et al. 2006). The paper presents the characterization and identification of the antigenic components of adult *T. canis* in the excretory-secretory product using the western blot technique.

Materials and methods

Parasite

Three pups of age group between 1–6 months were screened at random. They were examined for the natural infection of *T. canis* by faecal examination. The adult *T. canis* worms

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were then collected from the positive pups after deworming with a dose of piperazine citrate @ 220 mg/kg of body weight. The recovered worms were cultured in RPMI-1640 medium without supplementation of serum. The spent medium was collected at every 24 h up to 7 days.

Excretory-secretary (ES) antigens

The ES-antigens were prepared by ammonium sulphate precipitation method as described by Sugunan and Kaleysa (1986). Solid ammonium sulphate was slowly added into the above collected ES product to the point of saturation (761 g to 1 l of solution) and was centrifuged at $10,000\times g$ for 15 min. Pellet which was collected was suspended PBS, pH 7.4. The resuspended solution was dialysed to remove ammonium salt as checked by Nessler's reagent. Protein concentration was determined as per the method of Lowry et al. (1951).

SDS-PAGE analysis

The SDS-PAGE was carried out as per the method of Laemmli (1970) for characterization of excretory and secretory proteins.

Raising of hyper immune serum against TC-ES antigen

Two New Zealand white rabbits, weighing approximately 1.5 kg each, were used for raising of hyperimmune sera against TC-ES antigen. The immunization was done at the dose rate of 300 μ g of antigen mixed with equal volume of adjuvant per animal deep I/M. Excretory and secretory antigen of adult *T. canis* worm was emulsified in Freund's complete adjuvant (FCA) and used for primary immunization. Two successive booster doses were given along with Freund's incomplete adjuvant, 15 days apart. Rabbits were bled after 10 days of the first and second booster dose; serum was collected and stored at -20°C for further use.

Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was performed for detection of antibody response in rabbits experimentally immunized with TC-ES antigen using standard procedure as per Wijffels (1994). The intensity of color reaction was measured as absorbance in each well at 492 nm in an ELISA reader.

Western blotting

Western blotting was done to recognize the immunodominant polypeptides of TC-ES antigen using standard procedure of Burnette (1981) with slight modifications. The

resolved proteins were subsequently transferred to a nitrocellulose (NC) membrane. Blotting was carried out at 100 mA constant current for 3 h in the ATTO semi dry blot transfer machine, AE6670 (ATTO Corporation, Japan).

Development of blot

The NC paper was recovered after electrophoretic transfer and each lane was cut into strips. Successful transfer of the protein to the membrane was confirmed by staining the membrane with Ponceau's stain. Later, the strips were incubated with naturally infected dog sera diluted in PBS-T and the hyper immune sera raised against TC-ES antigen in rabbit diluted in PBS. Finally, they were incubated with goat anti-rabbit IgG-HRP and lately with rabbit anti-dog IgG-HRP, followed by development of blot using diaminobenzidine colour reagent system.

Results

SDS-PAGE analysis

Excretory and secretory antigens were visualized for protein bands following SDS. Protein bands ranging in size from 30 to 384 kDa, nine prominent bands of distinct sizes were observed at 384, 279, 187, 115, 79, 70, 57, 43 and 30 kDa (Fig. 1).

Fig. 1 SDS-PAGE analysis of excretory and secretory antigens of adult *T. canis*. Lane M: protein molecular weight marker. Lane 1: excretory and secretory antigens of adult *T. canis*

Enzyme linked immunosorbent assay (ELISA)

Indirect ELISA was performed with two rabbits hyperimmunised with TC-ES antigens and a high titre of IgG immunoglobulin response of 1:50,000 was observed (Fig. 2).

Western blotting

The specific reactivity of the *T. canis* ES-proteins was checked by western blotting. The immuno-reactivity of the naturally infected dog sera with the TC-ES antigens showed five bands at 43, 57, 105, 139 and 175 kDa. Out of these five bands, three (57, 139 and 175 kDa) were comparatively prominent bands (Fig. 3). The immuno-reactivity of the hyper immune serum raised in rabbits against TC-ES antigens was

Fig. 2 ELISA values of sera in rabbit immunized with *T. canis* ES antigens

Fig. 3 Western blotting showing immuno-dominant polypeptides reactive to *T. canis* infected dog serum. Lane M: protein molecular weight marker. Lane 1: excretory and secretory antigens of adult *T. canis*

Fig. 4 Western blotting showing immuno-dominant polypeptides reactive to anti-sera raised in rabbit against *T. canis* excretory and secretory antigens. Lane M: protein molecular weight marker. Lane 1: excretory and secretory antigens of adult *T. canis*

observed with ten polypeptides of 21, 25, 30, 37, 45, 50, 57, 69, 77 and 105 kDa. The six prominent bands were at molecular weights of 21, 30, 37, 57, 77 and 105 kDa (Fig. 4).

Discussion

In the present study, the *T. canis* ES antigens were characterized and nine prominent bands of distinct sized proteins were present at 384, 179, 115, 82, 79, 70, 57, 43 and 30 kDa were observed in SDS-PAGE analysis. Immuno-reactivity of these antigens with naturally infected dog serum showed five bands (43, 57, 105, 139 and 175 kDa), with three prominent bands of 57, 139 and 175 kDa. Like wise the immunoblot profile of TC-ES antigens with hyper immune serum raised in rabbits against TC-ES antigens showed ten bands 21, 25, 30, 37, 45, 50, 57, 69, 77 and 105 kDa, with six prominent bands (21, 30, 37, 57, 77 and 105 kDa). The immunoblotting profile of TC-ES antigens with positive dog and hyper immune serum raised in rabbits showed two common immunoreactive bands at 57 and 105 kDa, respectively.

Toxocara canis ES proteins have been characterized as mucins (glycosylated proteins of high molecular weight), 120, 40–45 kDa, lectins (sugar-binding proteins), 70 and 32 kDa as well as several other proteins at 26 and 55 kDa. There are molecular weight moieties observed in adult worms that may be related to the larval TC-ES products. A study by Badley et al. (1987) shows that TC-ES proteins from larvae, which may relate to those found in *T. canis* adults at molecular weight bands 34 (34.7), 53 (50.8), 57 (59.4), 66 (65.3), 76 (74.4) and 94 kDa (92.8 kDa) (Maizels et al. 2006). But there has not been a full characterization of the protein profile of adult *T. canis*.

Protein bands of adult worms recovered in this study are comparable with those reported by El-Massry (1999), (given in parenthesis) viz, 127.9 (125.37), 92.8 (90.00), 69.4

(69.25), 59.4 (58.36) and 48.4 kDa (46.57–47.13 kDa). The protein profile *T. canis* adult worm reported here also appear similar in molecular weight to those associated with larval *T. canis* proteins reported by El-Massry (1999) i.e. 127.9 (125.83), 74.4 (72.48), 65.3 (66.00–66.89), 59.4 (57.96) and 50.8 kDa (51.95 kDa).

In a study conducted by Nawal and Mona (2008) on serodiagnosis of human toxocariasis using adult antigens of *T. canis* an electrophoretic analysis of TC-ES antigens revealed eight polypeptide bands at 127, 94, 88.89, 50, 40.69, 33.77, 30 and 14.05 kDa, respectively.

Aida, (1999) reported that the SDS-PAGE profile of adult TC-ES antigens consist of eight bands (127.66, 94.30, 64, 58.81, 50.01, 46.61, 40.09 and 19.26 kDa). Immunoblot profile of TC-ES antigens with rabbit TC-ES anti-serum showed three common reactive bands (Mona et al. 2004). Morales et al. (2002) suggested that the 92 and 35 kDa polypeptides of *T. canis* second stage larvae E/S antigen are specific to *Toxocara* infection and the 57 kDa fraction of the larval *T. canis* E/S antigen (TC-ES-57) is specific to *T. canis* infection and does not cross react with sera of other related infections (Iddawela et al. 2007). In their study on the immunoblotting profile of TC-ES antigen, which when reacted with positive human and rabbit anti-TC-ES sera, showed five common immunoreactive bands at 47.46, 40.69, 30.53, 21.9 & 19.29 kDa. They suggested that 58.739 and 29.19 kDa polypeptides of the adult *T. canis* E/S are specific for *Toxocara* infection in human and these antigens merit further evaluation as candidates for use in the diagnosis of human toxocariasis.

In the present study, the immunoblotting profile of TC-ES antigen reacting with positive dog and hyper immune serum raised in rabbits against TC-ES antigen showed two common immunoreactive bands at 57 and 105 kDa. So these antigens merit further evaluation as candidate for use in diagnosis of toxocariasis in humans and adult dogs.

Acknowledgments The authors are thankful to the Director, Indian Veterinary Research Institute for providing all facilities to carry out the present investigation.

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