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ORIGINAL RESEARCH

Characterization of somatic antigens of adult *Toxocara canis* by western blotting

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Abstract

Aim: The objective of this study was characterize the somatic soluble antigens of adult *Toxocara canis* (Tc-SA) by western blotting.

Materials and Methods: *T. canis* worms were collected from the naturally infected pups after deworming. The somatic antigen was prepared as per standard procedure with slight modification. These antigens were separated using Sodium dodecyl sulphate-electrophoresis (SDS-PAGE). The specific reactivity of the Tc-SA proteins was checked against the serum of naturally infected dogs as well with the hyperimmune serum raised in the rabbit by western blotting.

Results: On SDS-PAGE recovered proteins ranged in size from 44 to 300 kDa. The immuno-reactivity of the naturally infected dog sera with the Tc-SA antigens showed 12 prominent immunoreactive bands of distinct sizes at 28.61, 32.60, 38.10, 43.04, 49.99, 67.57, 73.22, 105.77, 144.74, 161.11, 177.84 and 196.31 kDa. The immuno-reactivity of the hyper immune serum raised in rabbits against Tc-SA antigens was observed with 10 prominent bands of distinct sizes at 17.11, 24.15, 34.83, 43.46, 52.47, 55.89, 67.57, 70, 74.60 and 105.6 kDa.

Conclusions: Common antigens band were observed at 67 and 105 kDa. These antigens merit further evaluation as candidate for use in diagnosis of toxocarosis in humans and adult dogs.

Keywords: diagnosis, Somatic-antigens, *Toxocara canis*, western blotting

Introduction

Toxocara canis is a wide spread gastrointestinal nematode of dogs and other canids and is the causative agent of zoonotic disease in humans [1]. Larval stages of *T. canis* parasite have an obligatory tissue-migratory phase, giving rise to a drug resistant reservoir in dogs, and visceral larva migrans in humans [2,3].

Human infection is present worldwide and is a consequence of the habit of keeping dogs and cats for company, which favours the persistence of the parasite in the environment and its transmission [4]. In India, studies on the prevalence of human toxocarosis is very scarce and reports shows that its prevalence is 32.86% in Kashmir valley and 6.4 % in subjects residing in a rural area near Chandigarh [5]. In the other developing countries, this scenario is probably worse, Children playing in areas contaminated with dog faeces, as well as people with intimate contact with dogs, are subject to a higher risk of infection [6]. In the Developed countries like United States, human toxocarosis is the most common human parasitic worm infection and affects 13.9% of the population [7].

The cross-reactivity with antibodies from other endemic helminth infections is a limitation for the development of an accurate serodiagnostic test [Enzyme Linked Immunosorbent Assay (ELISA)] using the unfractionated excretory-secretory (ES) antigens from the *T. canis* second-stage larvae (TES Ag) [8]. In recent years, there have been important methodolo-gical advances in the diagnosis of many infectious diseases, but there are innumerable difficulties in the laboratory diagnosis of toxocarosis due to the laborious production of the antigens or the high costs of commercial diagnostic kits [9].

In the absence of parasitological evidence of infection, immunological methods are required for its diagnosis. The diagnosis of human toxocarosis depends on clinical and serological data because detecting Toxocara larvae by biopsy is difficult [10]. Since the development of the ELISA using ES antigens from TES Ag described by de Savigny et al. [11] and the subsequent western-blot method using the same antigen by Magnaval et al.[12], a general agreement has existed regarding the value of the TES Ag for the immunodiagnosis of human toxocarosis.

Recently number of diagnostic candidates have been investigated like *Toxocara* excretory-secretory antigen (TES-57) and recombinant *Toxocara*

excretory-secretory antigens (rTES-120, rTES-26, rTES-30USM) [13]. Presently a Dot-ELISA test for toxocarosis is under evaluation which is considered as a reliable one as it presents many advantages as a basic diagnostic test. For example, it is highly stable, it does not require specialised tools to analyse the results, it has a lower cost and it can be simultaneously applied to a large number of samples by a basically trained technical staff [14,15].

There are molecular weight moieties observed in adult worms that may be related to the larval TC-ES products [16]. The parasite proteins of this antigen which ellicit an immune response can be identified by immunological techniques. Therefore, this paper includes the identification and characterization of the antigenic components of adult *T. canis* present in the somatic soluble 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 were then collected from the positive pups after deworming with a dose of piperazine citrate @ 220mg/kg of body weight. The expelled worms were identified, washed three times with 0.9 % NaCl and frozen in 70% ethanol at - 40°C until further use.

Adult somatic antigens: The somatic antigen was prepared as per the Peixoto *et al*., with slight modification [17]. Whole somatic antigens were prepared by homogenization of adult worms in presence of cocktail of protease inhibitors, thereafter subjected for sonication at 8-10 micron peak to peak for 15 cycles with an interval of 30 sec. every time strictly maintaining the cold chain. The sonicated material was then centrifuged at 15000 rpm for 40 min at 4°C. The supernatant was collected thereafter and designated as soluble protein fraction of *T. canis* somatic antigen (Tc-SA) and kept in aliquots at -20°C for further experiment. Protein concentration was determined as the method of Lowry *et al*., [18].

SDS-PAGE analysis: The SDS-PAGE was carried out as per the method of Laemmli (1970) for characterization of somatic soluble proteins. [19].

Raising of hyper immune serum against Tc-SA antigen: Two New Zealand White rabbits, weighing approximately 1.5 kg each, were injected with Tc-SA at the dose rate of 300 µg per animal deep I/M. Somatic soluble antigen of adult *T. canis* was emulsified in equal volume of Freund's complete adjuvant (FCA) and used for primary immunization. Two successive booster doses were given along with Freund's incomplete adjuvant, 15days apart. Rabbits were bled after 10 days of the 1st and 2nd booster and the 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-SA antigen as per Wijfells *et al*. [20]. The intensity of colour reaction was measured as absorbance in each well at 492nm in an ELISA reader.

Western blotting: Western blotting was done to recognize the immunodominant polypeptides of Tc-SA antigen using standard procedure of Burnette [21]with slight modifications. The resolved proteins were subsequently transferred to a Nitrocellulose (NC) membrane. Blotting was carried out in ATTO semi dry blot transfer machine, AE6670 (ATTO Corporation, Japan)[21].

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-SA antigen in rabbit diluted in PBS-T. 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: On SDS-PAGE analysis, Tc-SA antigen revealed 14 protein bands ranging in size from 44 to 300 kDa, with a molecular moieties of 300, 182.17, 144.24, 105.31, 96.56, 93.97, 91.04, 83.34, 81.11, 79.47, 75.95, 71.45, 67.97 and 44 kDa (Fig-1).

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

Western blotting: The specific reactivity of the Tc-SA was checked by western blotting. The immuno-reactivity of the naturally infected dog sera with the Tc-SA antigens showed 12 bands at 28.61, 32.60, 38.10, 43.04, 49.99, 67.57, 73.22, 105.77, 144.74, 161.11, 177.84 and 196.31 kDa (Fig-3). The immuno-reactivity of the hyper immune serum raised in rabbits against Tc-SA antigens was observed with 10 polypeptides at 17.11, 24.15, 34.83, 43.46, 52.47, 55.89, 67.57, 70, 74.60 and 105.6 kDa (Fig-4).

Discussion

Protein bands of adult *T. canis* worm recovered in this study appear similar in molecular weight to those associated with adult *T. canis* proteins reported by El-Massry [22] at 91.04 (90), 67.97 (69.25) and 44 kDa (46.57– 47.13 kDa) [22]. Sun et al. 2007 described the SDS-PAGE protein profile of adult *T. canis* ranging in size from 3.4 to 325 kDa [23]. Protein bands of adult *T. canis* viz. 91.04, 67.97 and 44 kDa in the present study can be compared to those reported in the study of Sun et al., like 92.8, 69.4 and 48.4 kDa [23]. Nawal and Mona [24] reported the electrophoretic analysis of Tc-SA

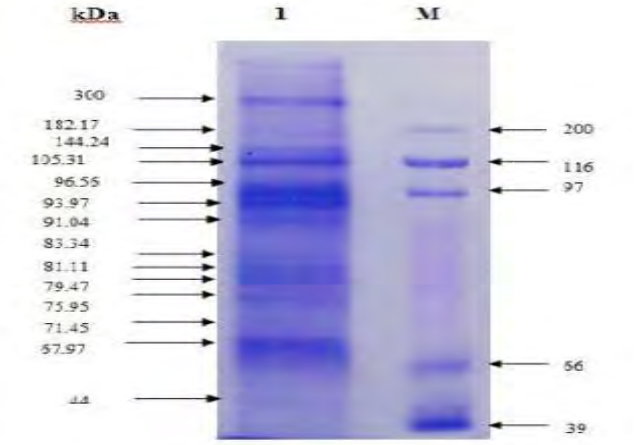

Fig-1. SDS-PAGE analysis of somatic antigens of adult *T. canis* Lane M: Protein molecular weight marker Lane 1: Somatic antigens of adult *T. canis*

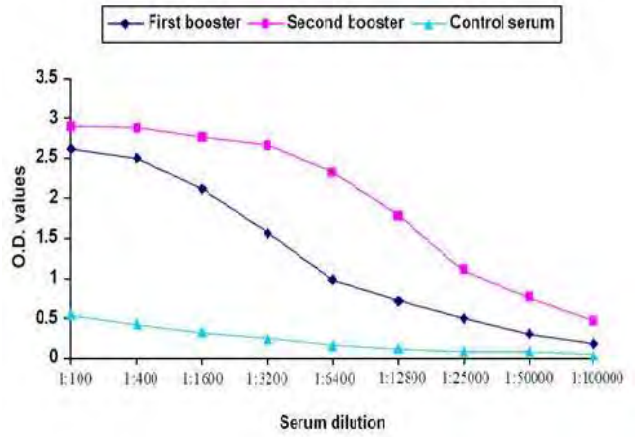

Fig-2. ELISA showing reciprocal serum dilution of rabbit, hyper-immunized with *T. canis* somatic antigens

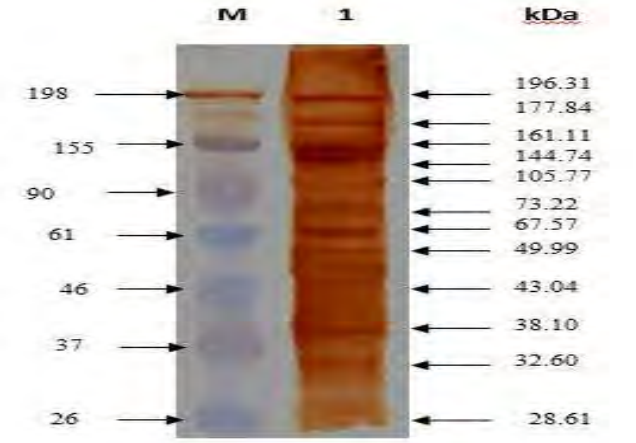

Fig-3. Western blotting showing immuno-dominant polypeptides reactive to *T. canis* infected dog serum Lane M: Protein molecular weight marker Lane 1: Somatic antigens of adult *T. canis*

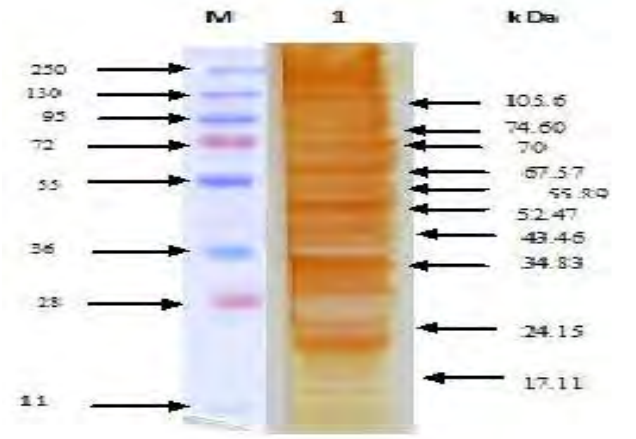

Fig-4. Western blotting showing immuno-dominant polypeptides reactive to hyper immune sera raised in rabbit against *T. canis* somatic antigens Lane M: Protein molecular weight marker Lane 1: Somatic antigens of adult *T. canis*

which revealed 13 polypeptide bands at 250, 125, 117, 90.43, 69.25, 56.76, 42.50, 40.69, 38, 35.70, 27.91, 21.98 and 19.29 kDa. Aida, [25] reported the SDS-PAGE profile of adult Tc-SA consisting of 7 (125.37, 117.73, 90.00, 69.25, 58.36, 47.13 and 46.53 kDa) protein bands.

Peixoto and co-workers [17] identified specific antigens from the adult stages of *T. canis* including 42, 58, 68 and 97 kDa that may be useful for the serodiagnosis of human toxocarosis. These antigens demonstrated considerable sensitivity and specificity and were considered as promising candidates for immunodiagnosis. The combination of the 58 and 68 kDa antigens was specific and sensitive for the anti-Toxocara antibody, supporting the possibility of using these two antigens for the immunodiagnosis of human toxocarosis in low-prevalent areas [17]. Further there are also reports that there is close taxonomic relationship between Ascaris sp. and Toxocara sp., and serological diagnosis there may be cross reactions as the two ascarids undoubtedly share antigens and epitopes [26].

We did a preliminary evaluation on the usefulness of soluble somatic antigens of adult *T.canis* as potential immunodiagnostic antigens. Compared to juveniles, adult worms may be a better source of antigens because they are relatively easier to obtain and handle. The immunoreactivity of the anti-sera raised against Tc-SA in rabbit was observed at 17.11, 24.15, 34.83, 43.46, 52.47, 55.89, 67.57, 70, 74.60 and 105.6 kDa and the immunoreactivity with the naturally infected dog sera was observed at approximately 28.61, 32.60, 38.10, 43.04, 49.99, 67.57, 73.22, 105.77, 144.74, 161.11, 177.84 and 196.31 kDa. We observed common immune dominant band at 67 and 105 kDa with both of hyperimmune and dog sera. These antigens merit further evaluation as candidate for use in diagnosis of toxocarosis in humans and adult dogs. However, detailed studies are required for characterizing the immuno-dominant polypeptides of the adult worm for their potential in the diagnosis of toxocarosis in dogs and in humans.

Conclusion

In the present study, we identified and characterized the somatic soluble antigens of *Toxocara canis* adult worm. We observed two common immuno-dominant bands at 67 and 105kDa. Further tests need to be performed to test whether these semi-purified adult *Toxocara* antigens might cross-react with other parasitic

infections, mainly those caused by nematodes, to further confirm the specificity of such antigens. Moreover the efficacy of the adult worm antigens should be tested to know whether these antigens are similar to the larval ES antigen, which is currently used as a standard diagnostic reagent. At last production of recombinant version of these antigens is needed which will not only standardize the routine diagnoses, but also allow the determination of the epidemiology of toxocarosis in humans and adult dogs through seroprevalence surveys.

Authors' contribution

SS designed the study, analysed and interpreted data, drafted the manuscript. SNR, SS and MDN collected, identified parasites and drafted manuscript. TKG helped in using his lab to carry out the work and he is also personally involved in carrying out the study. OKR, SCG and AK revised the manuscript. All authors read and approved the final manuscript.

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Competing interest

Authors declare that they have no competing interest.

References

1. Despommier, D. (2003) "Toxocariasis: clinical aspects, epidemiology, medical ecology, and molecular aspects". *Clin Microbiol Rev*, 16 (2): 265–272.
2. Vidal, J.E., Sztajnbok, J. and Seguro, A.C. (2003) Eosinophilic meningoencephalitis due to *Toxocara canis*: case report and review of literature. *American Journal of Tropical Medicine and Hygiene*, 69:341-343.
3. Hotez, P.J, Wilkins, P.P. (2009) Toxocariasis: America's most common neglected infection of poverty and a helminthiasis of global importance? *PLoS Negl Trop Dis* 3(3):e400.
4. Delgado, O. And Rodriguez-Morales, A.J. (2009) Aspectos clínico-epidemiológicos de la toxocariasis: una enfermedad desatendida en Venezuela y América Latina. *Bol Mal Salud Amb*, 1: 1-33.
5. Malla, N., Aggarwal, A.K. and Mahajan, R.C. (2002) A serological study of human toxocariasis in north India. *The National Medical Journal of India*, 15(3):145-47.
6. Espinoza, Y.A., Huapaya, P.H., Roldán, W.H., Jiménez, S., Arce, Z. And Lopez, E. (2008) Clinical and serological evidence of Toxocara infection in school children from Morrope district, Lambayeque, Peru. *Rev Inst Med Trop Sao Paulo*, 50: 101-105.
7. Jones, J.L., Kruszon-Moran, D., Won, K., Wilson, M. And Schantz, P.M. (2008) *Toxoplasma gondii* and Toxocara spp co-infection. *Am J Trop Med Hyg*, 78: 35-39.
8. Smith, H., Holland, C., Taylor, M., Magnaval, J.F, Schantz, P. and Maizels, R. (2009) How common is human toxocariasis? Towards standardizing our knowledge. *Trends Parasitol*, 25: 182-188.
9. Colli, C., Rubinsky-Elefant, G., Paludo, M., Falavigna, D., Guilherme, E. and Mattia, S. (2010) Serological, clinical and epidemiological evaluation of toxocariasis in urban areas of south Brazil. *Rev Inst Med Trop Sao Paulo*, 52: 69-74.
10. Roldán,W., Cornejo,W. and Espinoza,Y. (2006). Evaluation of the dot enzyme-linked immunosorbent assay in comparison with standard ELISA for the immunodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, 101(1): 71-74.
11. de Savigny, D.H, Voller, A. And Woodruff, A.W. (1979). Toxocariasis: serological diagnosis by enzyme immuno-assay. *J. Clin. Pathol*, 32: 284-288.
12. Magnaval, J.F, Fabre, R., Maurieres, P., Charlet, J.P and de Larrard, B. (1991). Application of the western blotting procedure for the immunodiagnosis of human toxocariasis. *Parasitol Res*, 77: 697-702.
13. Suharni, M., Norhaida, C.A. and Noordin, R. (2009). Development and Evaluation of a Sensitive and Specific Assay for Diagnosis of Human Toxocariasis by Use of Three Recombinant Antigens (TES-26, TES-30USM, and TES-120). *J. Clin Microbiol*, 47(6): 1712–1717.
14. Chieffi, P.P., Dos Santos, S.V., Queiroz, M.L. and Lescano, S.A. (2009) Human toxocariasis: contribution by Brazilian researchers. *Rev Inst Med Trop Sao Paulo* 51: 301-308.
15. María, V.B., Gioia, L.M., María, Á.L. and José, M.A. (2012) An evaluation of the dot-Elisa procedure as a diagnostic test in an area with a high prevalence of human *Toxocara canis* infection. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, 107(2): 194-197.
16. Maizels, R.M, Schabussova, I., Callister, D.M. and Nicoll, G. (2006) Molecular biology and immunology of *Toxocara canis*. In: Holland CV, Smith HV (Eds.) *Toxocara*: *The Enigmatic Parasite*. Cambridge MA 3–17.
17. Peixoto, P. L., Nascimento, E., Cançado, G. G. L., Miranda, R. R. C. and Rocha R. L. (2011) Identification of candidate antigens from adult stages of *Toxocara canis* for the serodiagnosis of human toxocariasis. *Mem Inst Oswaldo Cruz, Rio de Janeiro*, 106(2): 200-206.
18. Lowry, O.H., Rosebroug, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem*, 193: 265-275.
19. Laemmli, U. K. (1970) Cleavage of structural protein during the assembly of head of bacteriphage T4. *Nature*, 277: 680-685.
20. Wijffels, G. L. (1994) The secreted cathepsin L-like proteinases of the trematode, *Fasciola hepatica*, contain 3- hydroxyproline residues. *Biochem. J*, 299: 781–790.
21. Burnette, W.N. (1981). Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem*, 112(2):195-203.
22. El-Massry, A. A. (1999) Characterization of antigenic property of *Toxocara canis* and *Toxascaris* adults and larvae through immunodiagnosis electrophoresis (SDS-PAGE) and western blot technique. *J. Egypt Soc Parasitol*, 29: 335-45.
23. Sun,T., Bellosa, M. L., Lucio-Forster, A., Ho, D. S. and Bowman, D. D. (2007) A comparison of the protein constituents of the major body compartments of the dog roundworm, *Toxocara canis*. Veterinary Parasitology., 150 : 111–115.
24. Nawal, A.H. and Mona, S.M. (2008) Serodiagnosis of Human Toxocariasis Using Adult Antigens of *Toxocara canis. Research Journal Parasitology.*, 3 (3):85-91.
25. Aida, E.A. (1999) Characterization of antigenic property of *Toxocara canis* and *Toxascaris leonina* adults and larvae through immunodiagnostic electrophoresis (SDS-PAGE) and Western blot technique. *J. Egypt. Soc. Parasitol*. 29: 335-345.
26. Watthanakulpanich, D., Smith, H.V., Hobbs, G., Whalley, A.J. and Billington, D. (2008) Application of *Toxocara canis* excretory-secretory antigens and IgG subclass antibodies (IgG1-4) in serodiagnostic assays of human toxocariasis. *Acta Trop*, 106: 90-95.

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