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Identification and characterization of immunodominant antigen(s) of *Toxocara vitulorum* in buffaloes (*Bubalus bubalis*)

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ABSTRACT

Electrophoretic analysis of perienteric fluid antigen (PeAg), uterine antigen (UtAg), body wall antigen (BWAg) and whole worm extract antigen (WWEAg) showed 26, 23, 20 and 21 polypeptides in the range of 11.5-147.3, 20.3-147.3, 19.7-139.5 and 20.3- 126.3 kDa, respectively. Among the 4 antigens, 7 polypeptides of 20.3, 43.6, 53.1, 57.4, 66.9, 111.3 and 126.3 kDa were found to be common. The Western blotting analysis of PeAg, UtAg, BWAg and WWEAg, using high titre sera from heavily infected buffalo calves, revealed 5 immunodominant polypeptides of the mol. wt. 11.5, 66.9, 74.9, 105.5 and 126.3 kDa; 4 polypeptides of the mol. wt. 66.9, 83.4, 105.5 and 111.3 kDa; 6 polypeptides of 24.1, 33.7, 43.6, 57.4, 60.3 and 76.2 kDa; and 4 polypeptides of 30.4, 53.1, 57.4 and 66.9 kDa, respectively. The 66.9 kDa antigen was found to be immunodominant in 3 of the referral antigens (PeAg, UtAg and WWEAg) while 105.5 kDa antigen was immunodominant in PeAg and UtAg and a 57.4 kDa antigen in BWAg and WWEAg. Cross reactivity of *Haemonchus* spp. and *Paramphistomum epiclitum* antigens with *T. vitulorum* positive sera by western blotting revealed no cross reactive polypeptide and the 66.9 kDa antigen found in the somatic antigens of *T. vitulorum* may be a specific immunodominant antigen and could be further exploited for immunodiagnosis of latent toxocarosis.

Key words: buffalo, immunodiagnosis, somatic antigens, *Toxocara vitulorum*, Western blotting

Introduction

The diagnosis and control of *Toxocara vitulorum* is not easy as the larvae migrate in the host tissues, remaining as dormant or hypobiotic parasites. It is mainly transmitted through colostrums and milk, causing disease (severe anemia, diarrhoea, weight loss and anorexia) particularly in buffalo calves between 1 and 3 months of age (WICKRAMASINGHE et al., 2009). Only a limited number of studies have been published, as this parasite is more

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of a problem of bovine and buffalo calves, particularly from poor tropical countries. However, buffaloes are considered among the most productive domestic animals in these countries and are the major source of quality milk, with unique feed conversion capacity, and they produce milk more cheaply than cattle (STARKE-BUZETTI, 2006). India accounts for a significant share of the world's buffalo population, with nearly 55.30% of it (FAO statistic database, 2008 http://www.faostat.fao.org), where Punjab has around 6.5 per cent of India's buffalo population.

Toxocarosis can be diagnosed on the basis of clinical signs, necropsy findings, faecal examination for eggs and serological tests. However, coprological examination techniques fail to detect infections in adults and also during the prepatent stage and in mild infections in young calves. Various antigens, such as soluble extract antigen (Ex) from *T. vitulorum* larvae and adults, perienteric fluid antigen of adult parasite and crude antigen of adult *T. vitulorum,* have been used by several research workers (DE SOUZA et al., 2004; FERREIRA and STARKE-BUZETTI, 2005). Several serological tests such as IHA (Indirect Haemagglutination Test), CIEP (Counter-current Immuno Electrophoresis) and ELISA (Enzyme Linked Immuno-Sorbent Assay) have been applied for immunodiagnosis of *T. vitulorum* (BANERJEE et al., 1998; GHOSH and BANERJEE, 1998; SINGH et al., 2003) by using crude somatic antigens with variable results. Accordingly, the present study was conducted to identify specific immunodominant antigens for diagnosis of toxocarosis in buffaloes.

Materials and methods

Collection of parasites. Adult *T. vitulorum*, required for the preparation of different somatic antigens, were collected from buffalo calves found heavily positive for *T. vitulorum* eggs by the faecal floatation method, from the Rurka village of the Ludhiana district, Punjab, India. These selected animals were administered piperazine (100 mg/kg) orally once, followed by liquid paraffin (15-20 mL) after an 8-12 h interval to increase the intestinal motility and to facilitate the worm expulsion. After 24-36 h, *T. vitulorum* parasites were expelled with the faeces from the animals. These collected parasites were washed immediately with distilled water and normal saline solution repeatedly to remove the mucus and entangled excreta.

Serum collection. Blood was collected aseptically from buffalo calves (five) of 1 month of age, naturally and heavily infected with *T. vitulorum,* as diagnosed by faecal examination by the presence of large numbers of eggs. The sera was separated and stored in 1.5 mL aliquots at -20 °C until further use. Negative control sera from healthy buffalo calf (negative for *T. vitulorum* repeatedly by faecal examination) were collected.

Preparation of antigens. Somatic antigens (four) were prepared from the adult *T. vitulorum* by standard protocols. These included Perienteric Fluid antigen (PeAg), Whole

Worm Extract antigen (WWEAg), Body Wall antigen (BWAg) and Uterine antigen (UtAg).

Perienteric fluid antigen (PeAg). The posterior end of each parasite was punctured with the help of a hypodermic needle and the perienteric fluid was drained and collected in a sterile tube. The collected Perienteric fluid was centrifuged at 460g for 5 minutes and the supernatant was filtered through a 0.22 μm Millipore disposable syringe filter. The filtrate was aliquoted after the addition of phenyl methyl sulfonyl flouride (PMSF) at the rate of 10 μL /mL and was designated as Perienteric Fluid Antigen (PeAg) and stored at -20 °C until further use (AMERASINGHE et al., 1992).

Whole worm extract antigen (WWEAg). Toxocara vitulorum worms were dissected to remove the contents of the intestine and were homogenized in a sterile Teflon coated homogenizer in 0.01M PBS pH 7.2. Then sonication of this homogenized material was performed in Soniprep at 4 °C at 8μ peak for 2 min x 5 with a 1 minute interval, followed by centrifugation at 12,000 rpm for 1 hour at 4 °C. The supernatant was collected and filtered through 0.22 μm Millipore disposable syringe filters. The filtrate was aliquoted after the addition of PMSF, at the rate of 10 μL /mL, designated as whole worm extract Antigen (WWEAg) and stored at -20 °C (GOUBADIA and FAGBEMI, 1995).

Body wall antigen (BWAg). The worms were dissected to remove all the internal organs and the body walls thus obtained were homogenized in 0.01M PBS pH 7.2. Body Wall Antigen (BWAg) was prepared as per the method described above for WWEAg by GOUBADIA and FAGBEMI (1995).

Uterine antigen (UtAg). The uterus of female *T. vitulorum* worms filled with eggs were separated after dissection and used for preparation of the Uterine antigen (UtAg) as per the method of GOUBADIA and FAGBEMI (1995).

All antigens were stored after the protein was estimated by the method of LOWRY et al. (1951).

SDS-PAGE for characterization of antigens. All antigens (PeAg, UtAg, BWAg and WWEAg) were analyzed by discontinuous SDS-PAGE as described by LAEMMLI (1970) with slight modifications. The antigens were subjected to electrophoretic separation in 10 % resolving and 5% stacking gel in a vertical slab gel apparatus (16 cm x 16 cm x 1.5 mm) using a vertical electrophoresis system (Genei, Bangalore). Molecular weight markers of 14.3-97.4 kDa (Genei, Bangalore) were also run simultaneously at 120 V. The gels were subjected to Coomassie Brilliant Blue (CBB) stain for 4 h, followed by destaining and scanned by the gel documentation system (Syngene, USA).

Western blotting for identification of immunodominant polypeptides. The antigens subjected to SDS-PAGE were transferred onto 0.45 μm nitrocellulose membrane (NCM) using electrophoretic transfer apparatus at 100 mA for 90 min in a semidry blotting

apparatus (Atto Corporation, Japan) as described by TOWBIN et al. (1979). The transfer efficiency was checked by staining the NCM with 0.1% Ponceau solution. The NCM was incubated in blocking buffer containing 5% bovine serum albumin in phosphate buffered saline containing 0.05% Tween-20 (PBS-T). The NCM was washed 3 times (3-5 min each) with 0.01 M PBST (pH 7.4) and placed in primary sera (1:500) obtained from *T. vitulorum* naturally infected buffalo calves for 1 h at 37 °C. NCM were washed 3 times (3-5 min each) in 0.01 M PBST, to remove excess primary antibody, and incubated with rabbit anti-bovine IgG-HRPO conjugate for 1 h at 37 °C (1:1000) with intermittent gentle shaking. After washing, the NCM was then transferred to freshly prepared substrate solution (3, 3- diaminobenzidine tetra hydrochloride) and the colour was allowed to develop in the dark at room temperature for 10 min. The NCM was dried at room temperature and identification of the dark brown polypeptide bands developed was done by gel documentation system (Syngene, USA).

Cross reactivity. The Western Blotting (WB) technique was conducted for cross reactivity studies, utilizing somatic antigens of *Haemonchus* spp. and *Paramphistomum epiclitum* (obtained from Division of Parasitology, Indian Veterinary Research Institute, Izatnagar, India) and sera of buffalo calves naturally infected with *T. vitulorum* .

Results

Polypeptide profile of antigens. Somatic antigens, i.e. PeAg, UtAg, BWAg and WWEAg, showed 26, 23, 20 and 21 polypeptides in the range of 11.5 - 147.3, 20.3 - 147.3, 19.7 - 139.5 and 20.3 - 126.3 kDa, respectively (Table 1, Fig. 1). A number of polypeptides were found to be common among the different somatic antigens prepared from *T. vitulorum,* indicating sharing of common polypeptides, 7 polypeptides of 20.3, 43.6, 53.1, 57.4, 66.9, 111.3 and 126.3 kDa were found common among all the four antigens. A total of 16 polypeptides of 20.3, 23.2, 28.6, 41.4, 43.6, 49.2, 53.1, 57.4, 63.9, 66.9, 70.9, 96.4, 111.3, 126.3, 133.9 and 147.3 kDa among PeAg and UtAg, 10 polypeptides of 20.3, 43.6, 46.0, 53.1, 57.4, 60.3, 66.9, 80.1, 111.3 and 126.3 kDa between UtAg and BWAg and 13 polypeptides of 20.3, 24.1, 25.6, 33.7, 37.2, 43.6, 53.1, 57.4, 66.9, 105.5, 111.3, 126.3 and 133.9 kDa between PeAg and BWAg were found to be shared.

Identification of immunodominant polypeptides. The Western Blotting analysis of PeAg revealed 5 immunodominant polypeptides of the mol. wt. 11.5, 66.9, 74.9, 105.5 and 126.3 kDa using sera from buffalo calves naturally infected with *T. vitulorum*. Whereas UtAg, BWAg and WWEAg with the same sera revealed 4 immunodominant polypeptides of the mol. wt. 66.9, 83.4, 105.5 and 111.3 kDa; 6 polypeptides of 24.1, 33.7, 43.6, 57.4, 60.3 and 76.2 kDa; and 4 polypeptides of 30.4, 53.1, 57.4 and 66.9 kDa respectively. The same results were repeated with all five sera samples collected from different naturally infected buffalo calves. Negative control sera from healthy buffalo calves (negative for *T.*

vitulorum repeatedly by faecal examination) showed no reaction in the Western Blotting with the referred antigens (Fig. 2).

Table 1. Polypeptide profile of different somatic antigens of *T. vitulorum*

UtAg (kDa)	PeAg (kDa)	BWAg (kDa)	WWEAg (kDa)
147.3	147.3	139.5	126.3
133.9	139.5	126.3	111.3
126.3	133.9	111.3	105.5
111.3	126.3	105.5	99.0
96.4	111.3	99.0	90.9
90.9	105.5	80.1	74.9
86.9	96.4	76.2	66.9
83.4	74.9	66.9	60.3
80.1	70.9	60.3	57.4
70.9	66.9	57.4	53.1
66.9	63.9	53.1	46.0
63.9	57.4	46.0	43.6
60.3	53.1	43.6	40.9
57.4	49.2	40.9	37.2
53.1	43.6	37.2	33.7
49.2	41.4	33.7	30.4
46.0	39.3	25.6	26.9
43.6	37.2	24.1	25.6
41.4	33.7	20.3	24.1
30.4	28.6	19.7	22.2
28.6	26.9		20.3
23.2	25.6		
20.3	24.1		
	23.2		
	20.3		
	11.5		

Western blotting for cross reactivity study. Western Blotting utilizing somatic antigens of *Haemonchus* spp. and *Paramphistomum epiclitum* and sera of naturally infected buffalo calves with *T. vitulorum* revealed no cross reactive polypeptide. Thus, the immunodominant polypeptides of *T. vitulorum* identified in the study using the sera from naturally infected buffalo calves do not share any common antigen with those of *Haemonchus* spp. and *Paramphistomum epiclitum* and therefore could be called specific for *T. vitulorum*.

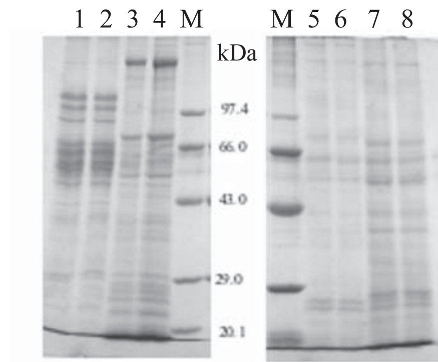

Fig. 1. Polypeptide profile of somatic antigens. Lane 1, 2-UtAg, Lane 3, 4-PeAg, Lane 5, 6-BWAg, Lane 7, 8-WWEAg, Lane M- Molecular weight marker.

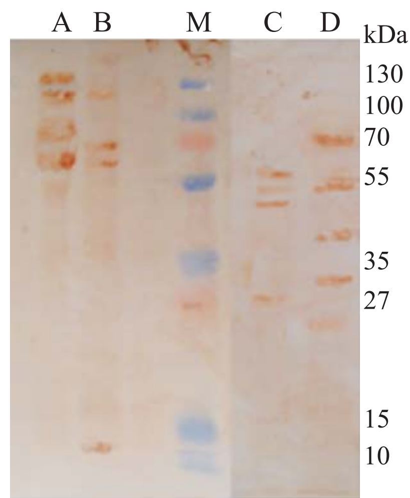

Fig. 2. Immunodominant polypeptides in different *T. vitulorum* antigens using sera of buffalo calf highly infected with *T. vitulorum*. Lane A-UtAg, Lane B-PeAg, Lane C-WWEAg, Lane D-BWAg, Lane M -molecular weight marker.

Discussion

Coprological demonstration of eggs for diagnosis of toxocarosis is not adequate in latent infections in adult animals, where the larval stages are arrested in the tissues and in pre-patent and mild infections in young calves. Therefore, alternative methods, viz. serological techniques, can prove to be promising for the diagnosis of toxocarosis in buffaloes. Detection of the sub-clinical disease will be useful in instituting chemotherapy against the dormant larvae and therefore the transmission cycle from the mother to the foetus will be disrupted, thus producing a significant impact on the economic aspect of the disease (ROBERTS, 2008).

STARKE-BUZETTI and FERREIRA (2004a, b and 2005) characterized adult soluble extract antigen (Ex) and perienteric fluid antigen (Pe) by SDS PAGE and Western Blotting, using immune sera and colostrum of buffaloes naturally infected with *T. vitulorum*. The results showed that the antigens Pe and Ex revealed 9 (11, 14, 31, 38, 58, 76, 88, 112 and 165 kDa) and 11 (11, 13, 16, 22, 25, 32, 43, 53, 68, 82 and 96kDa) protein bands by 12% SDS PAGE respectively. However, in the present study the Ex and Pe antigen showed a higher number of protein bands and the difference in the polypeptide profile encountered may be due to the difference in the gel concentration and the antigen preparation methodology.

Toxocara vitulorum infection can stimulate the immune system of buffalo. The antibodies against *T. vitulorum* present in buffalo cows are transferred passively to the calves through the colostrum (RAJAPAKSE et al., 1994). In calves, DE SOUZA et al. (2004) determined that passively acquired anti-ES, -Ex and -Pe antibody concentrations were highest 24 h after birth and remained at high levels until 45 days, the time coinciding with the peak of infection. Rejection of the worms occurred simultaneously with the decline of antibody levels. By WB, sera of buffalo calves at 1 day of age, after suckling the colostrum and at the beginning of infection, reacted with the same bands detected by serum and colostrum of the buffalo cows (STARKE-BUZETTI and FERREIRA, 2004a, b and 2005). Therefore, in the present study the sera from the naturally infected buffalo calves of 1 month of age used for the WB are an indication of the presence of the corresponding antibodies in the adult infected animals. The adult antigens utilized for the studies are more convenient than larval and ES antigens in terms of mass production, because of the easy availability of the adult worms.

Western Blotting analysis of PeAg, UtAg, BWAg and WWEAg, using sera from infected calves, revealed 5, 4, 6 and 4 polypeptides, of which most were of higher molecular weights, as reported earlier. It was interesting to record that the 66.9 kDa antigen was found to be immunodominant in 3 of the referral antigens (PeAg, UtAg and WWEAg) while 105.5 kDa antigen was immunodominant in PeAg and UtAg and a 57.4 kDa antigen in BWAg and WWEAg. Similarly, only bands of higher molecular weight antigen (76, 88, 112, 165 for Pe and 68, 82 and 96 for Ex) were detected by the anti-*T.*

vitulorum antibody in the sera of the calves at the peak of the infection, during the decline of EPG counts (rejection of the worms) and during the period of post rejection (STARKE-BUZETTI and FERREIRA, 2004a, b and 2005). Thus, antigens of high molecular weight (68-190 kDa) could represent useful larval antigens in reducing larval gut penetration and contribute to the inhibition of larval migration in buffalo cows.

FERNANDO et al. (1987) reported gel precipitating antibodies against an extract of embryonated *T. vitulorum* eggs in the serum and colostrum of some pregnant buffaloes and in the serum of their suckled calves. Also, anti- *T. vitulorum* antibodies against larval soluble antigen (STARKE-BUZETTI et al., 2001), extract (Ex) antigens and adult perienteric fluid antigen (DE SOUZA et al., 2004) were detected in the serum and colostrum of buffalo cows and calves naturally infected with *T. vitulorum.* Similarly, Immunoblot analysis using buffalo calf sera revealed 5 immunogenic components in the adult antigen, with molecular weights of 191, 166, 102, 65 and 54 kDa, whereas in the egg antigen polypeptides of 191, 105, 99 and 79 kDa were identified by ABDEL and ABDEL (2003).

The potential of immunodiagnostic assays for early detection of helminth infections is marred by non-specificity and cross-reactions, because of shared common antigenic epitopes in several helminths (GHOSH et al., 2005). From these results it can be concluded that the 66.9 kDa antigen present in the somatic antigens of *T. vitulorum* may possibly be a specific immunodominant antigen, as no cross reactivity was recorded with *Haemonchus* spp. and *P. epiclitum*. However, there is a need for further investigations to exclude all the possibilities of cross reactivity of this polypeptide with other related parasitic antigens. A similar 54 kDa immunogenic component of adult antigen has been reported earlier, as an immunogenic adult worm antigen by ABDEL and ABDEL (2003). Hence the 66.9 kDa polypeptide can be further purified by affinity chromatography techniques and screened against known sera samples, to determine the sensitivity and specificity of this polypeptide as a potent immunodiagnostic candidate antigen against latent toxocarosis in adult buffaloes. This would lead to chemotherapy by several available effective anthelmintics and thus control of the disease in adults and simultaneously in calves.

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SAŽETAK

Elektroforetska analiza antigena podrijetlom od perienteralne tekućine (PeAg), maternice (UtAg), te kutikule (BWAg) i somatskoga antigena (WWEAg) oblića *Toxocara vitulorum* pokazala je prisutnost 26 (PeAg), 23 (UtAg), 20 (BWAg) i 21 (WWEAg) polipeptida u rasponu od 11,5 do 147,3, od 20,3 do 147,3, od 19,7 do 139,5 i od 20,3 do 126,3 kDa. U četiri pretražena antigena najučestaliji su bili polipeptidi od 20,3, 43,6, 53,1, 57,4, 66,9, 111,3 i 126,3 kDa. Western blot analizom antigena podrijetlom perienteralne tekućine dokazano je pet imunodominantnih polipeptida molekularne mase 11,5, 66,9, 74,9, 105,5 i 126,3 kDa; podrijetlom od maternične tekućine četiri polipeptida molekularne mase od 66,9, 3,4, 105,5 i 111,3 kDa; podrijetlom od kutikule šest polipeptida molekularne mase od 24,1, 33,7, 43,6, 57,4, 60,3 i 76,2 kDa, dok se somatski antigen sastojao od četiri polipeptida mase od 30,4, 53,1, 57,4 i 66,9 kDa. Potvrđena je imunodominantnost antigena molekulske mase od 66,9 kDa za tri referentna antigena (PeAg, UtAg i WWEAg). Antigen od 105,5 kDa bio je imunodominantan u perienteralnoj tekućini i maternici dok je antigen od 57,4 kDa bio imunodominantan u kutikuli i iscrpku od cijeloga parazita odnosno kao somatski antigen. Treba istaknuti da antigen od 66,9 kDa nije pokazivao križnu reakciju s antigenima oblića *Haemonchus* spp. i *Paramphistomum epiclitum* što govori o njegovoj specifičnosti i mogućnosti njegova korištenja za imunodijagnostiku latentne toksokaroze.

Ključne riječi: bivol, imunološka dijagnoza, somatski antigeni, *Toxocara vitulorum*, Western blotting