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Research Article

CHARACTERIZATION OF EXCRETORY/ SECRETORY ANTIGEN OF *BUNOSTOMUM TRIGONOCEPHALUM* IN SHEEP

***S. Arunkumar**
**Department of Veterinary Parasitology, Madras Veterinary College*
Tamilnadu veterinary and animal sciences university, Chennai-600007, India
**Author for Correspondence*

ABSTRACT
In the present study, E/S antigen from *Bunostomum trigonocephalum* was prepared by *in vitro* culture method and hyper immune serum was raised against this antigen in rabbits. On SDS – PAGE analysis, the E/S antigen revealed six polypeptides at 21.0, 29.0, 47.0, 60.0, 94.0 and 101.0 kDa. Further, three immuno-reactive polypeptides at 29.0, 47.0 and 60.0 kDa were detected in western blotting. This antigenic characterization study will be helpful in developing suitable sero diagnostic in bunostomosis in sheep.

***Key Words**: Bunostomum trigonocephalum, E/S antigen, Western Blotting*

INTRODUCTION
Bunostomum trigonocephalum is an important hookworm in small ruminants in tropical areas. They inhabit in small intestine and cause progressive anaemia with associated changes in blood picture, hydraemia, oedema and stunted growth. Host immune response to helminthes is generally hampered by two main factors namely the complexity of antigenic profiles and the presence of cross- reactive determinants on antigens. To identify specific antigens, excretory/secretory antigens have received increasing attention recently. This is due to the fact that E/S antigens relatively display less complexity compared to somatic antigens. Further, the work on antigenic characterization of E/S antigen of *B.trigonocephalum* is scanty in India. Hence, the present investigation was undertaken to analyze the polypeptide profiles of E/S antigen of *B.trigonocephalum* for its use in serodiagnosis.

MATERIALS AND METHODS
Preparation of Antigen
Adult live *Bunostomum trigonocephalum* were collected from the small intestine of sheep slaughtered at corporation slaughter house, Chennai. The collected worms were washed five times in normal saline and subsequently washed five times in Phosphate buffered saline (PBS, pH 7.4), containing penicillin (500 IU/ml) and streptomycin (5mg/ml) and nystatin (1mg/100ml). Then, the worms were identified based on morphological features using standard keys (Soulsby, 1982).
The fresh and highly motile worms were transferred to RPMI 1640 medium containing penicillin (100 IU/ 100 ml) and streptomycin (1mg/100ml) and cultured at a concentration of approximately 400 worms per 20 ml in a culture flask at 5 per cent CO_2 atmosphere at 37°C for 24 hours. The medium was changed every 6 hours after incubation and fresh medium was added with 2 per cent glucose throughout incubation. Worm viability was monitored throughout this period on the basis of motility, integrity of the worms. Moreover, random samples of the culture fluid obtained during and directly after the incubation period were plated out on agar in order to exclude bacterial contaminations. After the incubation period, the culture medium was collected by decantation and filtered through a 0.22 μm filter (Millipore). Then, the culture medium was centrifuged at 10,000 rpm for 30 minutes at 4°C and the supernatant was labelled as excretory/ secretory (E/S) antigen. This culture procedure was repeated several times in order to obtain sufficient quantity of antigen. Finally, the antigen obtained was concentrated by dialysis (membrane cut off, 12 kDa) against polyethylene glycol (PEG 6000 – SRL, India) over a period of 6 hours. The

concentrated material was added with 1 mM phenyl methyl sulfonyl fluoride (PMSF) and 1 mM ethylene diamine tetra acetic acid (EDTA) to prevent proteolysis and stored at -20°C with 0.02 per cent sodium azide as preservative till further use. The protein concentration of the E/S antigen was determined using bicinchoninic acid (BCA) method (Smith *et al.,* 1985) using protein estimation kit (Genei, Bangalore).

Hyper Immune Serum

New Zealand white rabbits were immunized by intramuscular injection with E/S antigen (0.5mg) emulsified with Freund's complete adjuvant. Subsequently, three booster doses of antigen, emulsified with Freund's incomplete adjuvant were given at weekly intervals. Blood from the hyperimmunized rabbits was collected after 7 days of the last booster. Serum was separated and stored at -20^{o} C for using in the assay.

Antigen Characterization

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to observe the polypeptide patterns of E/S antigen of *B. trigonocephalum* according to their molecular weights under reducing gel conditions as per method of Laemmli (1970). The mini gel (12%) electrophoresis (Biorad, USA) was performed using 1mm thickness gel in a discontinuous system. The denatured antigen sample was loaded into each well with protein marker in a separate well. The gel was electrophoresed at a constant voltage of 120 till the tracking dye reached one cm above the lower extremity of the gel. After electrophoretic run, the gel was subjected to Coomassie brilliant blue (HiMedia, India) staining overnight with gentle agitation. After proper staining, the gel was put in destaining solution until clear demarcation of bands appeared.

The immuno-reactive polypeptides were identified by western blotting technique (Towbin *et al.,* 1979) using the hyper immune serum raised in rabbits. After the electrophoretic run, the nitrocellulose membrane was removed and was blocked in blocking buffer at 37°C for 2 hours. Then the membrane was washed in washing buffer three times with gentle agitation for 5 minutes each. The membrane was incubated in immune serum at a dilution of 1:100 in PBS for 2 hours at 37°C with gentle shaking. The nitrocellulose membrane was washed in washing buffer three times for 5 minutes each and immersed in solution of 1:1000 diluted anti-sheep IgG HRP conjugate (Sigma, USA) in PBS for 2 hours at 37°C. Then the membrane was washed three times in washing buffer for 5 minutes each. The membrane was treated with substrate solution (DAB) till the bands appeared.

RESULTS AND DISCUSSION

During incubation period, adult worms remained viable as assessed qualitatively by both motility and clumping tendency. No bacterial contamination was detected during *in-vitro* culture. The protein concentration of the E/S antigen was found to be 2.3 mg/ml. similar culture procedures were reported for isolating the E/S antigen by other workers (Anbu and Joshi, 2008; Karanu *et al.,* 1993). The protein concentration obtained in this study is in accordance with the reports of Jas *et al.,* (2010).

On Characterization the E/S antigen revealed six polypeptide bands at 21.0, 29.0, 47.0, 60.0, 94.0 and 101.0 kDa molecular weights by SDS-PAGE analysis. Out of six polypeptides, three polypeptides were identified as low molecular weights and another three were identified as medium range molecular weights. On western blot analysis, the E/S antigen probed with rabbit hyper immune serum showed three immuno-reactive polypeptides at 29.0, 47.0 and 60.0 kDa molecular weights. A very limited literature is available on antigenic profiles of *B. trigonocephalum* for comparison. Jas *et al.,* (2010) analysed the antigenic composition of E/S antigen of *B. trigonocephalum* by SDS- PAGE and observed ten polypeptides with molecular weights ranged between 22.5 to 98.0 kDa. Further, they demonstrated two immuno-reactive polypeptides at 39.0 and 50.0 kDa molecular weights in western blotting.

In recent years, E/S products of helminths have received increasing attention. This is due to the fact that E/S antigens usually display a relatively simple antigenic composition compared to the somatic antigen. Further, they also play numerous role in the host parasite interactions. The sensitivity and specificity of

E/S antigens were high compared to somatic antigens in immuno assays (Mir *et al.,* 2008). Hence, the antigenic composition of E/S antigen of *B. trigonocephalum* was analysed in this study.

Conclusion

In the present study, E/S antigen from *B. trigonocephalum* was prepared and hyper immune serum was raised against this antigen in rabbits. On antigenic characterization, the E/S antigen revealed six polypeptides at 21.0, 29.0, 47.0, 60.0, 94.0 and 101.0 kDa. On western blotting, three immuno-reactive polypeptides at 29.0, 47.0 and 60.0 kDa were detected. This study will be helpful in developing suitable serodiagnostic in bunostomosis in sheep.

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