Indian Journal of Animal Sciences **82** (9): 958–961, September 2012

Evaluation of serum antibody responses of Madras Red sheep to excretory/secretory antigen of *Haemonchus contortus*

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Received: 10 February 2011; Accepted: 20 February 2012

ABSTRACT

Excretory/secretory (E/S) antigen from *Haemonchus contortus* was isolated by culturing live adult worms in RPMI 1640 medium at 37° C for 24 h and the culture supernatant was used as antigen. Immuno blot analysis revealed that the E/S antigen showed 5 reactive bands at 24, 29, 46, 66 and 93 kDa. On immunization trial, sheep were immunized with 500 mg of E/S antigen along with montanide as adjuvant on day 0, 30 and 60 intramuscularly. Further, the assessment of serum antibody levels in immunized sheep was made by enzyme linked immuno sorbent assay (ELISA). Based on absorbance values, it was observed that the serum antibody levels were significantly higher up to 20 weeks post immunization in immunized sheep compared to control sheep.

Key words: Antibody, Excretory /Secretory antigen, ELISA, *Haemonchus contortus*, Sheep

Infections with *Haemonchus contortus* are a major constraint on sheep and goat health and production (Sood 1981) worldwide. Adult worms feed on blood resulting in poor growth rate and weight loss, and heavy infections can result in death (Newton and Munn 1999). Gastrointestinal nematodes control depends on the repeated use of anthelmintics and pastures management. There are concerns regarding drug residues in meat and the environment. Therefore, control of *H. contortus* infection through immunoprophylaxis would be an alternative strategy. Experimental vaccines against gastrointestinal nematodes have been divided into 2 classes. Antigens derived from the intestine of the parasite, are not exposed to the immune system during natural infection, whereas the natural or conventional antigens recognized during infection are generally excretory / secretory (E/S) antigens (Knox *et al*. 2003, Lejambre *et al*. 2008). Natural antigens should be equally effective against blood and non-blood-feeders and have the advantage that the immune response should be boosted by exposure under field conditions (Knox and Smith 2001). The E/S products have role in host tissue penetration, degradation of host proteins for nourishment, modulation of host immune response and prevention of blood clotting etc. (Karanu *et al*. 1993, Yatsuda *et al*. 2003 and Suchitra and Joshi 2005). Attempts were made to characterize E/S products of *H. contortus* as these substances could be potential target for immunological control of the disease. Work on *H.contortus* control using E/S antigens is scanty. Hence, the present study was undertaken to evaluate the efficacy of excretory / secretory antigens of *H. contortus* as vaccine candidate in the control of ovine haemonchosis.

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MATERIALS AND METHODS

Preparation of excretory / secretory antigens of Haemonchus contortus: Adult *Haemonchus contortus* worms were collected from abomasum of sheep slaughtered at corporation slaughterhouse, Perambur, Chennai. The collected worms were washed 5 times in normal saline and subsequently washed 5 times in phosphate buffered saline (PBS, pH 7.4), containing penicillin (500 IU/ml) and streptomycin (5 mg/ml). The worms were identified based on morphological features using standard keys (Soulsby 1982). After thorough washing, the worms were used for preparing the antigens.

The fresh and highly motile worms were transferred to RPMI 1640 medium containing penicillin (500 IU/ ml) and streptomycin (5 mg/ml) and cultured at a concentration of approximately 50 worms / ml in a culture flask at 5% CO_2 atmosphere at 37°C for 24 h. The medium was changed every 6 h after incubation and fresh medium was added with 2% 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. The culture medium was centrifuged at 10 000 rpm for 30 min at 4°C and the supernatant was labelled as excretory/ secretory (E/S) antigen. This culture procedure was repeated several times to obtain sufficient quantity of antigen. Finally, the antigen obtained was concentrated by dialysis (membrane cut off, 12 kDa) against polyethylene glycol over 6 h. The concentrated material was added with 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 1 mM ethylene diamine tetra acetic acid (EDTA) to prevent proteolysis and stored at –20°C with 0.02% 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.

Characterization of E/S antigen: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to observe the polypeptide patterns of E/S antigen of *H. contortus* according to their molecular weights under reducing gel conditions as per Laemmli (1970). The mini gel (12%) electrophoresis was performed using 1 mm thickness gel in a discontinuous system. Antigen sample (50 ml) was diluted with equal volume of sample buffer (1×) and boiled for 5 min in water bath for denaturation and was loaded into each well with protein marker in a separate well.

Identification of immunogenic fraction: The immunogenic fraction was identified by western blot analysis (Towbin *et al*. 1979) using mini trans-blot electrophoretic transfer cell. It was developed for transferring proteins from gel to nitro cellulose membrane (NCM) so that it can be detected with labelled antibodies. When protein is bound to a membrane, it is more accessible to specific detection methods than in the gel. After the electrophoretic run, the nitrocellulose membrane was removed and subjected to usual immunoblotting procedure.

Immunization trial: Immunization trial was conducted in sheep to evaluate the efficacy of E/S antigen of *H. contortus*. Madras Red breed of male sheep (12), 8- to 10- month-old were procured from Livestock Research Station, Kattupakkam, Tamil Nadu. All the sheep were maintained in clean animal shed and were fed with concentrate mixture (200g / animal), green fodder and water *ad lib.* during the research period. All animals were dewormed with ivermectin @ 0.2 mg/kg body weight 21 days prior to start of experiment. Sheep were divided into 2 groups, each group consists of 6 sheep. In group 1, sheep were immunized with 500 µg of E/S antigen along with adjuvant montanide ISA 206 on day 0, 30 and 60 through intramuscular route. In group 2, sheep were used as unimmunized control

Enzyme linked immuno sorbent assay (ELISA): Humoral immune responses in sheep immunized with E/S antigen was evaluated by monitoring the serum antibody levels by enzyme linked immuno sorbent assay (ELISA). Monospecific positive serum was obtained from ICAR scheme on "All India Network Programme on Gastro Intestinal Parasitism" at Department of Veterinary Parasitology, Madras Veterinary College, Chennai. Negative serum was collected from a healthy 2- to 3-week old Madras Red breed of lamb from Livestock Research Station, Kattupakkam (TANUVAS) Tamil Nadu. Test serum samples were collected at weekly intervals from all the sheep from 0 to 21 weeks of experimental period. The optimum concentration of antigen, serum and conjugate (anti-sheep IgG/HRP) were determined by checker board titration method using serial dilution of antigen, serum and conjugate. The optimum concentration of antigen (2 µg / ml), serum (1 in 200) and the conjugate (1 in 5000) was arrived by this method which was used in the test procedure.

Test procedure: ELISA was carried out as per Knox *et al*. (2003). The data obtained in the present study were statistically analyzed by ANOVA (Snedecor and Cochran 1968)

RESULTS AND DISCUSSION

In the present study, 10 000 live adult *Haemonchus contortus* worms were collected from the abomasum of sheep. During the incubation period, adult worms remained viable as assessed qualitatively by both motility and clumping tendency. No bacterial contamination was detected during *in vitro* culture. Similar culture procedures were reported by others for isolating the E/S antigen from *Haemonchus contortus* (Karanu *et al.* 1993, Schallig and Leeuwen 1997, Joshi and Singh 1999, Rathore *et al*. 2006, Anbu and Joshi 2008). The protein concentration of E/S antigen varied from 1.2 to 1.5 mg/ml in different batches.

On characterization of E/S antigen by SDS- PAGE revealed 5 polypeptide bands at 24, 29, 46, 66 and 93 kDa molecular weights (Fig. 1a). This finding is in partial agreement with the reports of Vervelde *et al.* (2001), Knox *et al.* (2005) and Rathore *et al.* (2006). On western blot

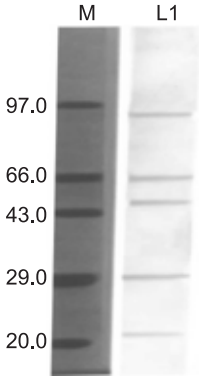

Fig. 1. Western blot analysis of E/S antigen using *H. contortus* infected sheep serum.M, Molecular weight marker; L1, total E/S antigen.

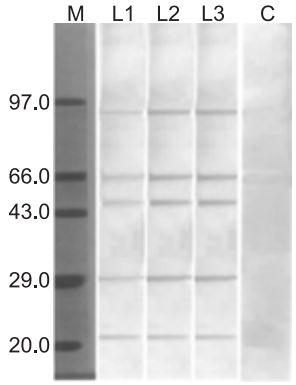

Fig. 2.Western blot analysis of sera collected from sheep immunized with total E/S antigen. M, Molecular weight marker; L1, total E/S antigen probed with serum collected from immunized sheep on day 30; L2, total E/S antigen probed with serum collected from immunized sheep on day 60; L3, total E/S antigen probed with serum collected from immunized sheep on day 90; C, control serum from unimmunized sheep.

analysis, the E/S antigen probed with serum from sheep experimentally infected with *H. contortus* showed 5 reactive bands at 24, 29, 46, 66 and 93 kDa (Fig.1b). Similarly, the E/S antigen probed with serum collected from sheep immunized with E/S antigen on day 30, 60 and 90 revealed 5 reactive bands at 24, 29, 46, 66 and 93 kDa (Fig.2). These findings are in accordance with the reports of Knox *et al.* (2005), Vervelde *et al.* (2001) and Rathore *et al.* (2006).

In this trial, sheep were immunized with 500 mg of E/S antigen along with montanide on day 0, 30 and 60 intramuscularly. The adjuvant used in the present study produced persistent antibody titres, without causing any tissue reactions at injection site. Several investigators conducted immunization trials with different dose schedule in sheep using E/S antigen. Further, the assessment of serum antibody levels were monitored in immunized and control sheep by ELISA. The mean absorbance values of ELISA from immunized and unimmunized control groups are presented in Table 1.

In immunized group, the mean absorbance values gradually increased from second weeks post immunization and reached a peak value of 0.70±0.02 on ninth week post immunization. The serum antibody levels were significantly ($P \leq 0.01$) higher up to 20 weeks post immunization in immunized group whereas, the mean absorbance values was low in control group. Schallig *et al.* (1994) observed significant elevation of serum antibody levels 2 weeks after second immunization in vaccinated sheep. Schallig and Leeuwen (1997) reported a constant increase in serum antibody responses 2 weeks after first immunization and subsequent immunization boosted the response. Similarly, a strong serum antibody titres were observed in sheep immunized with E/S antigen by Joshi and Singh (1999) and Vervelde *et al.* (2001). Knox *et al*. (2005) observed a gradual elevation of serum antibody responses 2 weeks after first immunization in vaccinated sheep compared to control sheep.

Table 1. Mean (+ SE) absorbance values of immunized and control sheep

Weeks post immunization	Group1 (immunized)	Group 2 (unimmunized control)
0	0.15±0.01	0.13±0.01
1	0.16±0.01	0.14±0.01
2	0.41±0.01	0.14±0.01
3	0.50±0.06	0.14±0.01
4	0.50±0.05	0.15±0.01
5	0.59±0.04	0.15±0.01
6	0.62±0.06	0.14±0.01
7	0.65±0.04	0.13±0.01
8	0.68±0.05	0.16±0.01
9	0.70±0.02	0.12±0.01
10	0.66±0.06	0.14±0.01
11	0.69±0.04	0.11±0.01
12	0.66±0.07	0.10±0.01
13	0.68±0.04	0.09±0.01
14	0.59±0.05	0.11±0.01
15	0.60±0.12	0.12±0.01
16	0.61±0.06	0.11±0.01
17	0.60±0.07	0.10±0.01
18	0.57±0.03	0.10±0.00
19	0.56±0.01	0.11±0.01
20	0.50±0.02	0.12±0.00
21	0.45±0.06	0.10±0.01

Analysis of variance

Source	df	F
Groups	1	1682.322**
Periods	21	14.7296**

** Highly significant ($P < 0.01$).

Based on the results, it was concluded that the E/S antigen induced significantly higher serum antibody levels till 20th week and thereafter gradual decrease noticed. This indicated that a booster dose is required after 20th week to maintain the antibody levels.

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