FLOW CYTOMETRIC ANALYSIS OF T – LYMPHOCYTE SUBSETS OF SHEEP IMMUNIZED WITH PURIFIED METABOLIC PRODUCTS OF OVINE ABOMASAL NEMATODE

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Abstract: In the present investigation live *Haemonchus contortus* worms were collected from abomasum of sheep and cultured in RPMI- 1640 medium at a concentration of 50 worms per millilitre in a culture flask at 37° C for 24 hours. The culture medium was collected and centrifuged at 10,000 rpm for 30 minutes at 4°C and the supernatant was used as antigen. Further, the antigen was purified by thiol-sepharose affinity chromatography and characterized by immunoblotting . On immunization trial, sheep were divided into two groups, each group consists of six animals. In group-I, sheep were immunized with 500 μg of purified antigen whereas, in group-II was used as unimmunized control. Assessment of lymphocyteproliferative responses was determined by flow cytometry. Based on the results, it was observed that the mean percentage of $CD4^{+}$ T-lymphocyte subsets was significantly higher in purified antigen immunized groups compared to control group.

Keywords: *Haemonchus contortus,* Lymphocytes,sheep.

Introduction: *Haemonchus contortus* is a major blood feeding nematode of sheep and goats world wide. Infection can lead anaemia, oedema, emaciation and gastric disturbances resulting principally from blood loss. Heavy infections may be fatal especially in young animals. Current methods of this parasite is through periodic use of anthelmintic drugs and pasture management. However,the emergence of resistant worm populations to anthelmintics has accelerated the need for the development of alternative strategies to prevent the adverse effects of this nematode.Vaccination seems to be the ultimate, effective and sustainable strategy for controlling infection in small ruminants. Hence, the present study was carried out to analyse T-lymphocyte subsets in sheep immunized with purified metabolic antigen of *Haemonchus contortus* using flow cytometry assay.

Materials and Methods:

Preparation of antigen: Fresh adult *Haemonchus contortus* worms were collected from abomasum of sheep slaughtered at local abattoir, Chennai. After thorough washing in phosphate buffered saline (PBS, P^{H} 7.4), the fresh live worms were transferred to RPMI – 1640 medium containing penicillin (500 IU/ml) and streptomycin (5mg/ml) and cultured at a concentration of approximately 50 worms per ml in a culture flask at 37°C for 24 hours. The medium was changed every 6hours of incubation with fresh medium. After incubation the culture medium was collected and centrifuged at 10,000 rpm for 30 minutes at 4° C and the supernatant was used as metabolic antigen. The protein content was determined using bicinchoninic acid method [10]. Further, the antigen was purified by thiol-sepharose affinity chromatography [5]. The purified antigen was characterized using sodium dodecy1 sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to analyse the polypeptide profile [7]. Further the immunogenic fraction was identified using western blotting [12].

Experimental trial: Immunization was conducted to assess T-lymphocyte subsets in sheep. Twelve Madras red breeds of male sheep aged around 6 months old were maintained in clean environment. They were fed with concentrate mixture (200 g per animal), green fodder and water *ad libitum* during experimental period. All sheep were dewormed with fenbendazole at the dose rate of 5mg / kg body weight 21 days prior to start of the trial. Sheep were divided into two groups, each group consists of 6 sheep. In group-I, sheep were immunized with 500μg of purified antigen along with montanide ISA 206 as adjuvant on day 0,30 and 60 through intra muscular route. In group-II, sheep were used as unimmunized control. All experimental animals were maintained well as per the guidelines issued by the institutional animal ethical committee (IAEC). The heparinised blood samples were collected at 15 days interval from all sheep to access the T-lymphocyte subsets. A total of 5000 infective larvae (L3) of *H. contortus*was given orally to all sheep on 90th day after immunization as challenge infection.

Analysis of T-lymphocyte subsets: Lymphocytes subsets in peripheral blood of immunized sheep were determined using flow cytometry assay [13]. Five millilitres (5ml) of heparinised blood samples were collected from the jugular vein of all sheep at 15 days interval during experimental period. Two millilitre of blood was diluted with an equal volume of PBS (P^{H} 7.4) and carefully layered over an equal volume of Histopaque solution (specific gravity, 1.077). Then, the tubes were centrifuged at 2,500 rpm for 30 minutes at room temperature. After centrifugation,

the white ring of cells (interface) were separated, washed twice with ice cold PBS and washed once with FACS buffer. The cell counts were adjusted to l x 10^6 cells in 100 µl of PBS.

A single colourimmuno fluorescence staining procedure was followed as per Pena *et al*. (2006). The lymphocytes were incubated with monoclonal antibody specific for mouse anti - ovine CD4 (l:100) and mouse anti- ovine CD8 (l:100) at 4° C for 2 hours in dark room. The cells were then incubated with goat anti mouse IgG- FITC conjugate (1:50 dilution) at 4° C for one hour in dark room .The cells were washed twice with ice cold PBS and finally 500 µl of FACS buffer was added. After the final wash, the cells were resuspended in 500 µl of FACS buffer for immediate analysis. Further, the cellswere also fixed in FACS buffer containing 4 per cent paraformaldehyde until it was read in flow cytometer. The stained cells were analysed using FACScan flow cytometer equipped with Cell Quest software (Becton Dickinson, USA).The data obtained in the present study were statistically analysed by ANOVA [11].

Results and Discussion: In the investigation, a total of 10,000 live worms were collected for preparation of antigen. During incubation, adult worms remained viable as assessed by motility and clumping tendency. The protein concentration of purified antigen was 1.8 mg/ml. On western blot analysis, the purified antigen probed with serum from sheep infected with *H. contortus* showed a single immuno reactive band at 66 kDa (Fig – 1). These findings are in accordance with the reports of earlier worker[6],[9]. The mean percentage of $CD4^+$ T- lymphocyte subsets of purified antigen immunized and control animals are presented in Table -1. In group-I, there was gradual increase in mean percentage of $CD4^+$ T-lymphocyte subsets (28.21±2.49 to 47.17±1.36) from 4-16 weeks post immunization and there after gradual decrease was noticed. The mean percentage of $CD4^+$ T-lymphocyte subsets was high (47.17±1.36) at 16th weeks after immunization. Whereas, the mean percentage of $CD4^+$ T- lymphocyte counts was significantly lower in unimmunized control group. But it was also observed that there was a mild increase in $CD4^+$ T – lymphocyte counts from 12 to 16 weeks in control animals due to challenge infection. Further, the mean percentage of $CD8^+$ T-lymphocyte subsets of purified antigen immunized and control group are presented in Table -2.It was observed that there was no significant increase in mean percentage of $CD8^+$ T- lymphocyte subsets in immunized and control animals throughout the study period.

Similar finding were reported by many workers. Reference[4] shows that an increase in $CD4^+$ T-lymphocyte counts in immunized sheep and also suggested that antibody works synergistically with $CD4^+$ T-lymphocytes to confer immunity. Reference[2] shows an increase in the mean percentage of $CD4^+$ T- lymphocytes in peripheral blood of vaccinated sheep compared to control. Reference[3] shows an increase in $CD4^+$ T-lymphocyte counts in peripheral blood of immunized sheep compared to control group. There was an increase in E/S specific lymphocyte proliferative responses in vaccinated sheep[1]. Reference[8] shows that lambs depleted of their $CD4^+$ T- lymphocyte were more susceptible to *H. contortus* infection than undepleted lambs.

In thepresent study, it was observed that the mean percentage of $CD4^+$ T – lymphocyte subsets was significantly higher in purified antigen immunized groups compared to control group throughout the period. The mean percentage of $CD4^+$ T- lymphocyte subsets was high (47.17±1.36) at 16th week after immunization. Whereas, there was no significant increase in the mean percentage of $CD8^+$ T-lymphocyte subsets in immunized and control group. Based on the results, it was concluded that $CD4^+$ T-lymphocyte subsets play a significant role in inducing protective immunity in immunized sheep.

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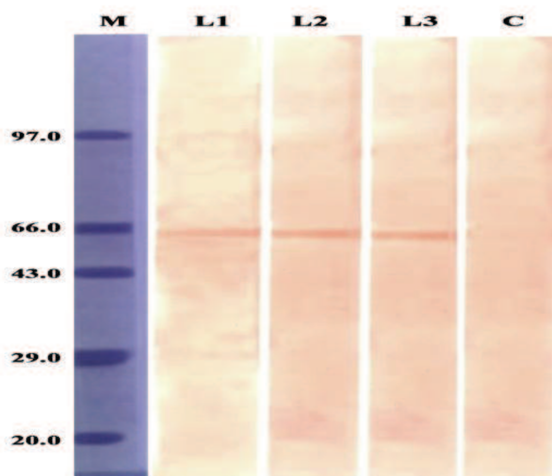
Table – 1 Mean (± S.E) percentage of $CD4^+$ T- lymphocyte subsets of purified antigen immunized and control sheep

Weeks Post immunization	Group – I (immunized)	Group – II (Unimmunized control)
0	21.52±1.17	18.26±2.15
2	26.53±3.21	20.14±2.14
4	28.21±2.49	19.28±4.16
6	30.62±2.14	20.67±3.96
8	32.54±2.42	21.36±2.96
10	34.82±4.50	20.56±4.12
12	38.45±3.27	21.16±3.92
14	42.67±2.96	20.32±2.84
16	47.17±1.36	21.16±3.94
18	36.28±4.94	22.34±3.64
20	26.18±6.47	20.74±2.16
22	23.35±3.20	19.27±3.12

Table – 2 Mean (± S.E) percentage of $CD8^+$ T- lymphocyte subset of immunized and control sheep

Weeks Post immunization	Group – I (immunized)	Group – II (Unimmunized control)
0	10.34±0.68	10.27±0.31
2	11.25±0.71	12.15±0.83
4	10.42±0.37	10.98±1.01
6	12.14±0.51	12.52±0.72
8	11.62±1.61	10.44±0.24
10	12.12±1.31	12.12±0.35
12	12.50±1.22	12.64±0.29
14	11.98±1.27	11.12±0.64
16	11.23±0.78	12.08±0.77
18	12.47±0.51	11.74±0.69
20	11.87=0.91	12.12=0.61
22	12.13=0.73	11.35=0.71

Plate -6



analysis of purified E/S antigen of*Haemonchuscontortus*
M – Molecular weight marker
L1,L2,L3 – Purified E/S antigen probed with *Haemonchuscontortus i*nfected sheep serum
C – Control serum from unimmunized sheep

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