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# Identification of Immunoreactive Polypeptides in *Haemonchus contortus* by Immunoprecipitation during Prepatent Infection in Sheep

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**ABSTRACT**

In the current study immunoreactive polypeptides in Adult Somatic Extract (ASE), Excretory Secretory Product (ESP) and Larval Somatic Extract (LSE) of $L_3$ stage were identified by employing immunoprecipitation, a lesser utilized technique on helminth parasites. Prepatent polyclonal sera were used to capture polypeptides in immunoprecipitation. Polypeptides eluted from the immune complex in ASE in SDS-PAGE were 26, 60, 120 & 170 kDa whereas in ESP these were 26, 32, 60 and 120 kDa showing absence of 170 kDa and presence of 32 kDa in ESP. In LSE the polypeptides captured were 26, 32, 60 & 120 kDa. Possible functional significance of the peptides in different antigens has been discussed and development of an immunoprophylactic agent has been speculated.

**Keywords:** *Haemonchus contortus,* immunoprecipitation, immunoreactive polypeptides, polyclonal sera

Development of vaccine against gastrointestinal nematodes (GINs) is an urgent requirement for sustainable worm control programmes. *Haemonchus contortus* is the most pathogenic GIN in tropical and subtropical countries. It is a blood sucking nematode which attaches with the help of a buccal lancet to the mucosa of abomasum. As estimated, a single worm can suck 0.05 to 0.07 ml blood/day (Clark *et al*. 1962; Malviya *et al*. 1979). Larval stage particularly $L_4$, immature and mature worms suck blood and in young animals causing severe anaemia leading to death. Pathophysiological effects also occur in infected animals which decrease productivity in terms of milk and fleece. Simpson (2000) described parietal cell dysfunction in infected animals raising abomasal pH.

For the last two decades attempts are being made to identify immunogens which are suitable for immunoprophylaxis and has been reviewed extensively (Knox *et al*. 2001). These molecules have been derived from several sources including somatic extract, surface antigens, excretory-secretory product and other native antigens of larva and adult parasite. During the last decade, concealed or hidden antigens have been employed for immunization which includes H11 and H-gal-GP with encouraging results, but low efficacy with recombinant ones. Now most of the workers are of the opinion that single polypeptide as an immunogen is not sufficient to elicit protection in the natural host. Therefore, a cocktail of immunogenic polypeptides will be required for desired protection. Immunoprecipitation can give firsthand knowledge about the polypeptides reacting to immune serum; recombinant form of those polypeptides may be of help in future to produce recombinant cocktail vaccines. Further, colostral transfer of immunity indicates that immunoprotection against *H. contortus* is antibody mediated (Smith *et al*. 1999).

With this view, somatic extract of adult *H. contortus*, its excretory secretory product and larval somatic extract from $L_3$ were utilized during the study to identify immunoreactive polypeptides with the help of prepatent polyclonal sera raised in experimentally infected sheep to identify polypeptides reacting to anti-*H. contortus* antibodies during prepatency. Immunoprecipitation was utilized to isolate and concentrate polypeptides from the parasite products containing several polypeptides.

Immunoprecipitation is also very useful for identification of a desired polypeptide present in lower concentrations.

## MATERIALS AND METHODS

### Procurement of adult *H. contortus*

Adult *H. contortus* were procured from the abomasa of sheep from local abattoir at Bareilly, Uttar Pradesh (India) and carried to the laboratory in PBS. The worms were identified as per the characteristics described by Yamaguti (1961).

### Coproculture of *H. contortus* $L_3$ larvae

100 gm faeces of sheep was triturated, dried in sunlight, sterilized and autoclaved. Female adult *H. contortus* worms collected were homogenized for collection of eggs. The eggs were spread over the sterilized faeces on a petridish and incubated at room temperature for 7 days for the harvesting of $L_3$ larvae which were identified as described by Van Wyk *et al*. (2004).

### Experimental sera

Experimental sera against *H. contortus* were raised in adult sheep (n=6) below one year age and similar body weight which were infected *per os* with 10,000 $L_3$ of *H. contortus.* Two sheep of similar age and weight were kept as control. Pre-infection sera were collected followed by post infection sera at weekly intervals which were stored at -20ºC. The animals maintained in the animal shed only and not allowed to graze to ascertain negativity for *H. contortus* infection. All the procedures have been carried out in accordance with the guidelines laid down by the IAEC.

### Antigens

i. Adult somatic extract (ASE): Adult *H. contortus* collected from abomasa of sheep were washed with several changes of PBS. Somatic extract was prepared by homogenizing worms in PBS and further sonicated (@50 Hz for 15 cycles). Homogenate was centrifuged at 10,000 rpm for 30 minutes at 4°C. Supernatant was collected and filtered through 0.2 µm filter membrane and stored at -70°C with protease inhibitor cocktail.
ii. Excretory-Secretory Products (ESP): 500 adult worms were washed thrice in PBS supplemented with 1,000 U/ml Penicillin and 1mg/ml Streptomycin and incubated at 37°C in a medium consisting of RPMI-1640 supplemented with 2% glucose, HEPES L-glutamine and Penicillin/Streptomycin 100 µl/ml and 100 µg/ml, respectively. Medium was changed after four hours. The worms were incubated overnight in fresh medium. After incubation the worms were removed, the media were pooled and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was collected and filtered through 0.2 µm filter membrane. Protease inhibitor cocktail was added to the filtrate and stored at -70°C.
iii. Larval Somatic Extract (LSE): Larvae were centrifuged at 10,000 rpm and debris removed by washing with slightly warmed PBS. 500 µl packed volume of larvae were homogenized in PBS. After complete homogenization the suspension was sonicated (@50 Hz for 15 cycles). The sonicated larvae were treated with B-PER solution (Pierce) for lysis and maximum protein extraction. The suspension was centrifuged at 10,000 rpm for 40 min at 4°C and supernatant was collected. The protein present in the supernatant was measured and stored at -20°C.

### Protein estimation

Protein was estimated as per the method of Lowry *et al*. (1951) in different samples.

### SDS-PAGE

ASE, ESP and LSE of *H. contortus* were fractionated by SDS-PAGE following method of Laemmli (1970). A mini vertical slab gel electrophoresis was used with 12% polyacrylamide gel containing 0.1% SDS. The samples were mixed with sample buffer in a proportion of 1:1. The quantity of protein loaded per track was 10 µg. The samples were heated in boiling water bath for 2 min before loading. Mini vertical slab gel was run at 100 V for 2 hrs and then stained with coomassie brilliant blue R-250 (0.1%). The molecular weight marker (PageRuler® Prestained Protein Ladder, Fermentas®) was run parallel along with sample protein to determine the relative molecular weight of the peptides.

#### Immunoprecipitation (IP)

Antigen capture and formation of immune complex was performed as per manufacturer's protocol for Immunoprecipitation kit (Pierce®). In this technique prepatent sera (pooled 1 & 2 week) were utilized. The kit was provided with Immunoprecipitation plate having wells coated with protein G.

Formation of immune complex: Protein G-coated plate was washed with IP buffer (450 ml of PBS and 50 ml of different surfact-Amps X-100). For polypeptide capture, prepatent sera from sheep was mixed with IP buffer in 1:1 ratio (100 µl sera and 100 µl buffer), put into the wells and incubated at room temperature (22-25°C) for 2 hrs. Each well of the plate was washed with IP buffer five times. Later, antigen mixed with IP buffer in the ratio of 1:1 and 100 µl was added to each well and incubated overnight at 4°C for formation of immune complex. Finally, the plate was washed five times with IP buffer to remove excess protein.

SDS-PAGE of captured polypeptides: Antigenic polypeptides captured on the plate were eluted from immune complex by adding the elution buffer (30 µl/well) and collected within a minute. The polypeptides thus collected were mixed with 20 µl of 1X non-reducing sample buffer and 5 µl of neutralizing buffer (Pierce®). The prepared sample was fractionated by SDS-PAGE. 50 µl of the sample was loaded per well and the gel run at constant voltage (100 V). After complete run, the gel was stained with 0.1% coomassie brilliant blue G-250 for 20 min and destained immediately.

Controls for immunoprecipitation: As per the manufacturer's instructions, one well each was incubated with antigen and antibody only, as controls.

Positive control in the Immunoprecipitation was experimental sera of those animals which were experimentally infected with *Haemonchus* larvae and further positive for *Haemonchus* eggs after prepatency (21-32 days PI). Serologically negative sera were collected from uninfected controls which were negative for *Haemonchus* eggs after prepatency.

## RESULTS

#### Protein estimation

The protein concentration estimated by Lowry method was 6.3 mg/ml in the ASE, 1.2mg/ml in the ESP and 0.8mg/ml in LSE.

#### SDS-PAGE

SDS-PAGE analysis of the ASE revealed prominent polypeptides of 15, 26, 40-50, 60 and 100 kDa. Three polypeptides above 100 kDa (120 kDa, 170 kDa and a polypeptide above 170 kDa) were also fractionated (Figure 1).

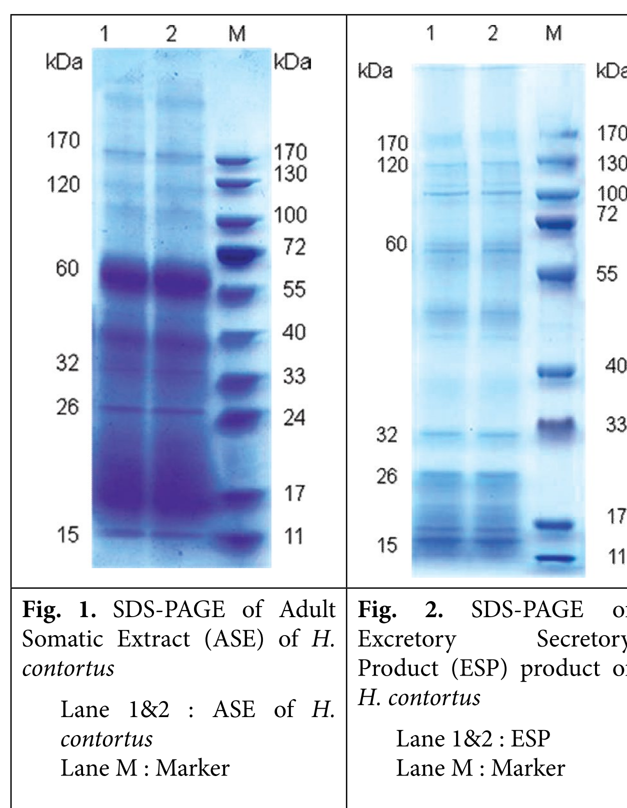


**Fig. 1.** SDS-PAGE of Adult Somatic Extract (ASE) of *H. contortus*

Lane 1&2 : ASE of *H. contortus*
Lane M : Marker

**Fig. 2.** SDS-PAGE of Excretory Secretory Product (ESP) product of *H. contortus*

Lane 1&2 : ESP
Lane M : Marker

SDS-PAGE of ESP of *H. contortus* revealed several polypeptides. More prominent polypeptides were 15, 26, 32, 50, 60, 100, 120 and 170 kDa (Figure 2).

LSE fractionated in to polypeptides ranging between 10-200 kDa. More prominent polypeptides were 17, 22, 24, 26, 28, 30, 32, 34, 55, 58, 66, 95, 100, 110, 120, 170 and a band greater than 170 kDa (Figure 3).

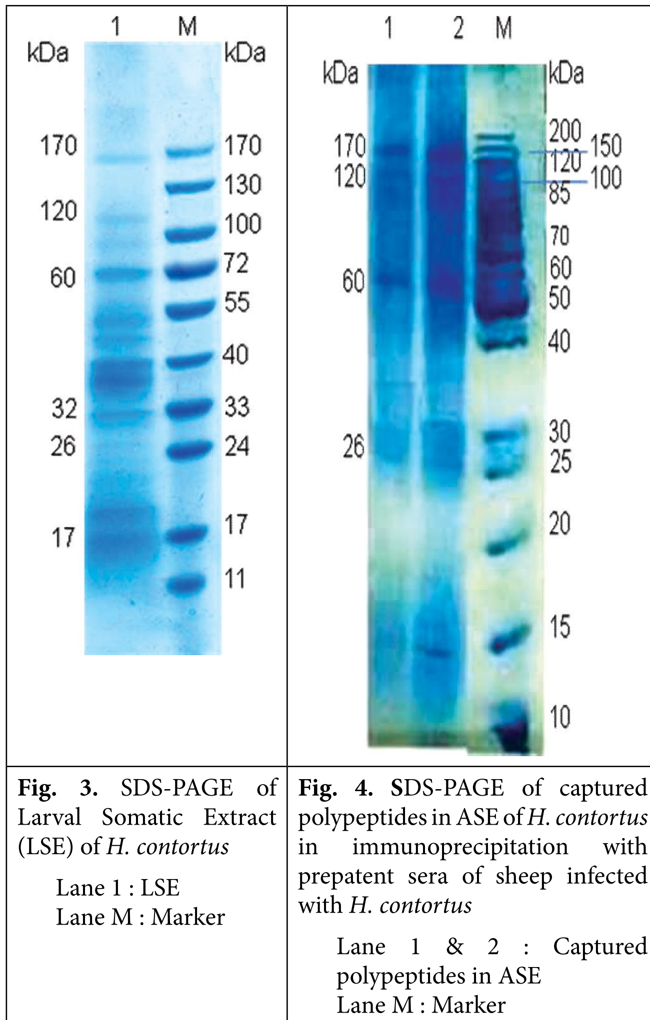


**Fig. 3.** SDS-PAGE of Larval Somatic Extract (LSE) of *H. contortus*

Lane 1 : LSE
Lane M : Marker

**Fig. 4.** SDS-PAGE of captured polypeptides in ASE of *H. contortus* in immunoprecipitation with prepatent sera of sheep infected with *H. contortus*

Lane 1 & 2 : Captured polypeptides in ASE
Lane M : Marker

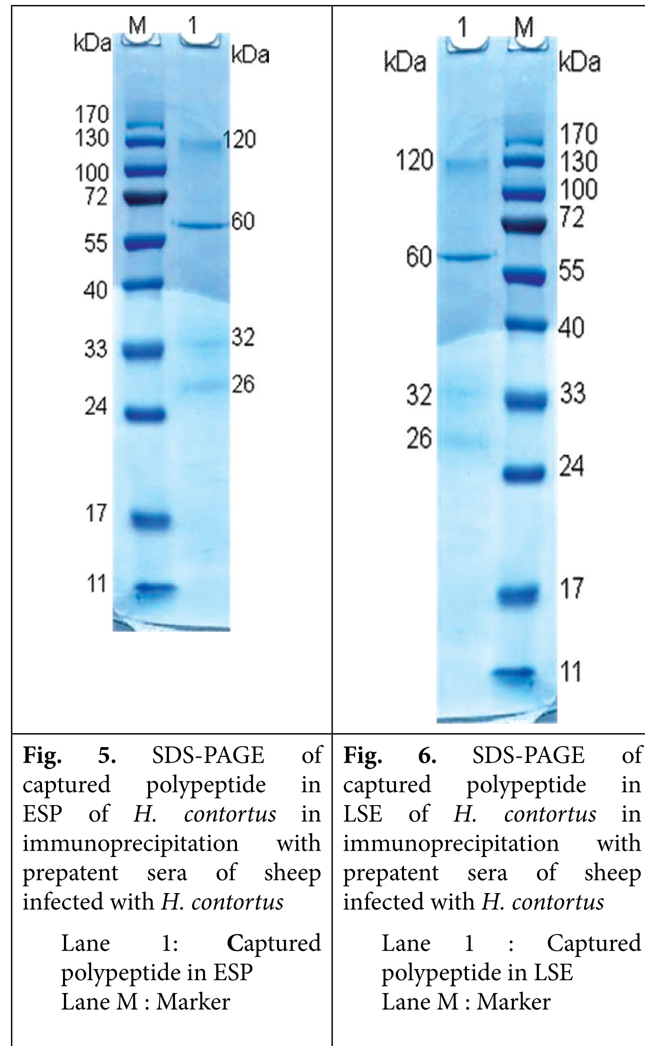


**Fig. 5.** SDS-PAGE of captured polypeptide in ESP of *H. contortus* in immunoprecipitation with prepatent sera of sheep infected with *H. contortus*

Lane 1: Captured polypeptide in ESP
Lane M : Marker

**Fig. 6.** SDS-PAGE of captured polypeptide in LSE of *H. contortus* in immunoprecipitation with prepatent sera of sheep infected with *H. contortus*

Lane 1 : Captured polypeptide in LSE
Lane M : Marker

### Immunoprecipitation

SDS-PAGE of captured polypeptides: SDS-PAGE of captured polypeptides of ASE by polyclonal prepatent sera in immunoprecipitation revealed recognition of 26, 60, 120 and 170 kDa polypeptides as well as some faint bands below 60 kDa (Figure 4).

SDS-PAGE of captured polypeptides of ESP through immunoprecipitation revealed recognition of 26, 32, 60,120 kDa polypeptides, by polyclonal prepatent sera (Figure 5).

Captured polypeptides in LSE with polyclonal prepatent sera were 26, 32, 60 & 120 kDa showing reactivity to prepatent sera (Figure 6).

SDS-PAGE with control sera did not reveal any polypeptides in SDS-PAGE.

## DISCUSSION

With the view that the polypeptides in *H. contortus* derived products (ASE, ESP and LSE) may react to antibodies present in the polyclonal sera, immunoprecipitation was employed to identify polypeptides reacting to anti-*H.*

*contortus* antibodies in prepatent polyclonal sera. Bankov *et al*. (2002) suggested utilization of immunoprecipitation as a method to resolve less abundant species of polypeptides in a sample. Polypeptides were identified in ASE, ESP and LSE of *H. contortus* in immunoprecipitation. The technique proved suitable to identify the polypeptides reacting to antibodies in the experimental prepatent sera. The results of immunoprecipitation indicate that anti-*H. contortus* antibodies captured polypeptides in ASE, ESP and LSE during prepatency. Humoral immune response as assessed by ELISA (Schallig *et al*. 1994; Schallig and Van Leeuwen, 1999) and dot ELISA (Prasad *et al*. 2007) indicate that anti-*H. contortus* antibodies react to polypeptides in different antigens. Coyne and Brake, (2001) described that in natural infections of various parasitic organisms immune responses were directed against a number of antigenic fractions. Based on the available literature, functional significance of the polypeptides proteases is discussed below.

During the present study, the 26 kDa polypeptide may be glutathione s-transferase (GST) which is supported by findings of other workers. In *H.contortus*, presence of GST having molecular weight of 23, 23.5 & 24 kDa has already been reported (Sharp *et al*. 1991). Gomez-Munoz *et al*. (1996) found a 26 kDa polypeptide in the adult *H. contortus* in purified fraction utilizing gel filtration and anion exchange chromatography. Moreover, in western blotting, a 26 kDa polypeptide of ASE and LSE have been found to react with anti-GST antibody indicating presence of GST in the adult as well as larva of *H.contortus* (Prasad *et al*. unpublished). Polypeptides in the range of 32-40 kDa in *H. contortus* have been found to be cysteine proteases (CPs). Antibodies reacting to 32 kDa polypeptide of ESP may be against CP which needs further investigation. A 60 kDa polypeptide was captured in ASE, ESP as well as LSE by polyclonal antibodies in prepatent sera. Earlier, Prasad *et al*. (2007) found a 60 kDa polypeptide to be an immunodominant polypeptide in western blotting which reacted to monospecific sera of sheep infected with *H. contortus*. In the present study 60 kDa is present in ASE as well as ESP which may be gut derived polypeptide reported to be secreted in ESP.

An additional polypeptide in the ASE is 170 kDa which was absent in ESP. Although, 170 kDa has been designated to be a component of H-gal-GP, presently we do not know whether the immunoprecipitated polypeptide is component of H-gal-GP, but its absence in ESP indicates that, it may be a polypeptide associated with gut epithelium and is not secreted.

Results of Immunoprecipitation studies indicated 26, 32, 60 & 120 kDa polypeptides in $L_3$, adult somatic and ES product of *H. contortus*. In our opinion a combination of native polypeptides suspected to be proteases or other enzymes may be tried for immunoprotection studies as well as biochemical determination for enzymatic nature.

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